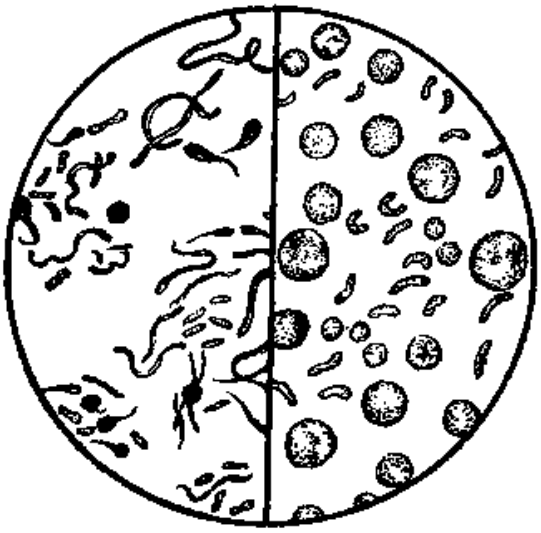
I.I. Generalov



MEDICAL MICROBIOLOGY, VIROLOGY & IMMUNOLOGY

Part 2
Medical Bacteriology & Medical Virology

Lecture Course
for Students of Medical Universities

VITEBSK STATE MEDICAL UNIVERSITY

2016

Ministry of Health of the Republic of Belarus
Higher Educational Establishment
“Vitebsk State Order of Peoples' Friendship Medical University”

I.I. Generalov

MEDICAL MICROBIOLOGY, VIROLOGY & IMMUNOLOGY

Part 2
Medical Bacteriology & Medical Virology

Lecture Course
for Students of Medical Universities

VITEBSK
2016

УДК [579+616.31]=111(07)
ББК 52.64 я73+56.6 я73
Г 34

Printed according to the decision of
Educational&Methodological Consociation on Medical Education
(July 1, 2016)

Reviewed by:
D.V.Tapalsky, MD, PhD, Head of Microbiology, Virology and Immunology Dpt,
Gomel State Medical University

Microbiology, Virology and Immunology Dpt,
Belarusian State Medical University, Minsk

Generalov I.I.

Г 34 Medical Microbiology, Virology and Immunology. Part 2. Medical Bacteriology & Medical Virology – Lecture Course for students of medical universities / I.I. Generalov. – Vitebsk, - VSMU. - 2016. - 392 p.

ISBN 978-985-466-743-0

The Lecture Course on Medical Microbiology, Virology and Immunology accumulates a broad scope of data covering the most of essential areas of medical microbiology. The textbook is composed according to the educational standard, plan and program, approved by Ministry of Education and Ministry of Health of the Republic of Belarus. This edition encompasses all basic sections of the subject – General Microbiology, Medical Immunology, Medical Bacteriology and Virology. Part 2 of the Lecture Course comprises Medical Bacteriology and Medical Virology sections.
The book is directed for students of General Medicine faculties and Dentistry faculties of higher educational establishments.

УДК [579+616.31]=111(07)
ББК 52.64 я73+56.6 я73

ISBN 978-985-466-743-0

CONTENTS

ABBREVIATION LIST

Ab – antibody
ACE 2- angiotensin- converting enzyme 2
Ag – antigen
AIDS – acquired immunodeficiency syndrome
ALT – alanine aminotransferase
ANUG – acute necrotizing ulcerative gingivitis
BCG vaccine – bacillus Calmette-Guerin vaccine
BCYE agar – buffered charcoal yeast extract agar
BSE – bovine spongiform encephalopathy
BSK medium – Barbour-Stoenner-Kelly medium
BSL – biosafety level
CADIS – candida-associated denture induced stomatitis
CagA – cytotoxin-associated gene A
cAMP – 3',5'-cyclic AMP
cccDNA – covalently closed circular DNA
CJD – Creutzfeldt-Jakob disease
CMV – cytomegalovirus
CNS – central nervous system
CRISPR – clustered regularly interspaced short palindromic repeats
CRS – congenital rubella syndrome
CSF – cerebrospinal fluid
DAT – diphtheria antitoxin
DFA-TP – direct fluorescent-antibody test for *T. pallidum*
DIC – disseminated intravascular coagulation
DPT vaccine – diphtheria, pertussis, and tetanus vaccine
DST – Diaskintest
DtxR – diphtheria toxin repressor
EAEC – enteroaggregative E. coli
EBV – Epstein-Barr virus
EF – edema factor
EHEC – enterohemorrhagic E. coli
EIEC – enteroinvasive E. coli
ELISA – enzyme-linked immunosorbent assay
EMB agar – eosin-methylene blue agar
EPEC – enteropathogenic E. coli
ESBL – extended-spectrum beta-lactamase
ETEC – enterotoxigenic E. coli
EVD – Ebola virus disease
FFI – fatal familial insomnia
GCF – gingival crevicular fluid
GPAC – gram-positive anaerobic cocci

GSS – Gerstmann-Straussler-Scheinker syndrome
HAART – highly active antiretroviral therapy
HAV – hepatitis A virus
HBsAg – hepatitis B surface antigen
HBV – hepatitis B virus
HCV – hepatitis C virus
HDV – hepatitis D (delta) virus
HEV – hepatitis E virus
HHV-6 – human herpesvirus type 6
HHV-7 – human herpesvirus type 7
Hib – *Haemophilus influenza* type b
HLA – human leukocyte antigen(s)
Hsp – heat shock protein(s)
HSV – herpes simplex virus
HUS – hemolytic uremic syndrome
ICTV – International Committee on Taxonomy of Viruses
IGRA – interferon-gamma release assay
KSHV – Kaposi's sarcoma-associated herpesvirus
LBAT – liver bile acid transporter protein
LCV – legionella-containing vacuole
LF – lethal factor
LOS – lipooligosaccharide
LPS – lipopolysaccharide
M cells – microfold cells
MAPK kinase – kinase of mitogen-activated protein kinase
MAT – microscopic agglutination test
MDR *M. tuberculosis* – multidrug resistant *M. tuberculosis*
MDT – multidrug therapy
MERS – Middle East respiratory syndrome
MODS – multiple-organ-dysfunction syndrome
MOMP – major outer membrane protein
MPA – meat peptone agar
MPB – meat peptone broth
MRSA – methicillin-resistant S*taphylococcus aureus*
MSF – Mediterranean spotted fever
NAATs – nucleic acid amplification tests
nAChR – nicotinic acetylcholine receptor
NP – nucleoprotein
NSAID – nonsteroidal anti-inflammatory drug(s)
NSP – non-structural protein
OMP – outer membrane protein(s)
Osp – outer surface protein(s)
PA – protective antigen

PBP – penicillin-binding protein
PCR – polymerase chin reaction
PEP – post-exposure prophylaxis
PRNP – prion protein gene
PRP – polyribosil ribitol phosphate
pYV – plasmid of yersinia virulence
RDS – respiratory distress syndrome
RMSF – Rocky Mountain spotted fever
RPR test – rapid plasma reagin test
RT-PCR – reverse transcription PCR
RV – rubella virus
SARS – severe acute respiratory syndrome
SFG – spotted fever group
SIRS – systemic inflammatory response syndrome
SLT – Shiga-like toxin
SPI – salmonella pathogenicity island
STD – sexually transmitted disease
STX toxin – Shiga toxin
T3SS – type III secretion system
T4SS – type IV secretion system
T7SS – type VII secretion system
TBEV – tick-borne encephalitis virus
TCBS agar – thiosulfate-citrate-bile-sucrose agar
TCP – toxin-coregulated pili
TSEs – transmissible spongiform encephalopathies
TSST – toxic shock syndrome toxin
TST – tuberculin skin test
UNAIDS – Joint United Nations Programme on HIV/AIDS
VacA – vacuolating cytotoxin A
VAP – ventilator-associated pneumonia
VBNC forms – viable but non-culturable forms
VDRL test – Venereal Disease Research Laboratory test
VP – viral proteins
VPI – *Vibrio* pathogenicity island
VZV – varicella-zoster virus
WHO – the World Health Organization
XDR *M. tuberculosis* – extensively drug-resistant *M. tuberculosis*
Yop proteins – yersinia outer proteins

MEDICAL BACTERIOLOGY

Chapter 1

CAUSATIVE AGENTS OF SUPPURATIVE, WOUND AND HOSPITAL-ACQUIRED INFECTIONS

PATHOGENIC STAPHYLOCOCCI

The History of Discovery

R. Koch discovered staphylococci in 1878. L. Pasteur obtained the pure culture of these bacteria in 1880. Later they were thoroughly studied by F. Rosenbach (1884).

Classification of Staphylococci

Staphylococci pertain to the family *Staphylococcaceae*, genus *Staphylococcus.* The genus *Staphylococcus* comprises more than 40 species. Three most common species are *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Staphylococcus saprophyticus*.

S. aureus is one of the major human pathogens. It causes suppurative lesions in different tissues, wound infections, food poisoning, septicemia and many other serious disorders.

Other staphylococci are usually the representatives of normal human microflora of skin and mucosal tissues. Nevertheless, *S. epidermidis* affects immunocompromised patients and patients with implanted appliances (intravenous catheters, drains, etc.)

The infections of prosthetic devices can be also caused by *S. hominis* and *S. haemolyticus*. In rare cases *S. saprophyticus* is able to cause urinary tract infections predominantly in young women.

The rest of staphylococcal species (*S. schleifeiri, S. warneri, S. capitis* and many others) is not regarded as causing infections in humans.

Structure and Properties of Staphylococci

Morphology

Staphylococci are small spherical microbes, 0.5-1 μm in diameter. Microbial cells are usually grouped into irregular ***grape-like*** clusters but

single cells, diplococci, and short microbial chains can be readily observed. Under unfavorable conditions they may turn into L-forms.

Staphylococci are ***gram-positive*** non-motile bacteria without flagella and spores. They can form capsule especially in the host tissues during infection.

Cultivation

Staphylococci easily grow on basic nutrient media at 37°C and pH of 7.2-7.4. During cultivation they are capable of producing water-insoluble pigments – golden (mostly, by *S. aureus*), gray (*S. epidermidis*), and white or yellow (*S. saprophyticus*). Pigment synthesis is facilitated in milk-supplemented media.

Egg yolk salt agar containing up to 10% of NaCl is applied as selective medium for culture of staphylococci. The elevated concentrations of NaCl inhibit the growth of concomitant bacteria.

All staphylococci produce pigmented smooth convex glistening colonies of medium sizes. When growing upon egg yolk agar, *S. aureus* renders positive *lecithinase activity*, degrading egg yolk lecithin. In most of cases other staphylococcal species don't express lecithinase.

Mannitol salt agar with egg yolk is also used as selective medium for staphylococci with additional detection of mannitol fermentation.

Selective *Baird-Parker agar* for isolation of *S. aureus* contains lithium chloride and egg yolk tellurite enrichment, which prevent the growth of other bacteria. After overnight incubation *S. aureus* demonstrates shiny convex black colonies resulting from tellurite reduction.

Staphylococci can cause hemolysis of rabbit, sheep and human erythrocytes on blood-containing media.

In liquid nutrient media staphylococcal cultures develop diffuse opacity.

Biochemical properties

Staphylococci are facultative anaerobes. They ferment carbohydrates yielding acid metabolites (e.g., lactic acid) without gas. These bacteria utilize proteins with hydrogen sulfide production.

Staphylococci (predominantly *S. aureus*) liquefy gelatin, coagulate milk, and reduce nitrates to nitrites. Also they produce catalase that differentiates them from streptococci as well as urease, phosphatase and some other enzymes.

Coagulase production distinguishes *S. aureus* from other members of the same genus (with rare exceptions). Thus, *S. aureus* refers to as

coagulase-positive bacteria, while other staphylococci are ***coagulase-negative***.

Likewise, *S. aureus* express *thermostable* ***nuclease***.

Antigenic structure

Staphylococci possess antigenic polysaccharides and proteins in peptidoglycan of microbial cell wall and microcapsule.

Cell wall teichoic acids carry additional antigenic determinants of staphylococci.

Virulence factors

S. aureus expresses a great variety of virulence factors, including exo- and endotoxins. Most of them are plasmid-controlled; some may be under chromosomal control.

S. aureus produces α-, β-, γ- and δ-hemolysins.

Alpha-hemolysin or α-toxin has the lethal, necrotic and hemolytic activity. It is ***pore-forming toxin***, capable of embedding into the target cell membrane with subsequent membrane impairment. This toxin readily lyses rabbit erythrocytes, damages platelets and smooth muscle cells, etc. It is lethal for rabbits on injection.

β-Toxin renders ***sphingomyelinase*** activity. It damages the membranes of human erythrocytes and many other cells.

γ-Hemolysin can affect erythrocytes of many mammalian species as well as white blood cells (neutrophils and macrophages).

δ-Hemolysin damages cytoplasmic membranes of various mammalian cells. It is able to aggregate within membrane lipid bilayer, thereby forming membrane channels that mediate cell lysis.

Poreforming ***leukocidin*** (or *Panton-Valentine* toxin) destroys leukocytes and bone marrow precursors of blood cells during infection.

S. aureus synthesizes more than 10 variations of heat-stable ***enterotoxins***, causing food poisoning. They are resistant to intestinal proteolytic enzymes.

Enterotoxins show high biological capacity, activating great subset of T-lymphocytes. The latter is followed by redundant proinflammatory cytokine production by T cells and macrophages (IL-1, IL-2 IL-6, IL-12, alpha-TNF, etc.) Cytokine release provokes systemic inflammation with severe tissue damage.

Enterotoxins affect mostly the gastrointestinal tract that results in vomiting and diarrhea.

Toxic shock syndrome toxin (***TSST***) resembles in structure enterotoxins B and C. It can induce ***toxic shock syndrome*** especially in menstruating women that used absorbing tampons. Tampons can be contaminated by TSST-producing staphylococci. TSST has strong superantigenic activity that finally may result in systemic shock with fever, collapse, desquamative skin rashes and multi-organ dysfunction.

Similar action is promoted by staphylococcal ***exfoliative toxins*** (ETA and ETB). Exfoliatins are absorbed from the skin primary infection site and carried by the blood stream to the large areas of the skin. They destroy deep cellular layers of the epidermis resulting in ***staphylococcal scalded skin syndrome***. This disease affects mainly newborn infants and may be fatal. More than 50% of skin area can be damaged. The skin becomes red, wrinkled, and large blisters filled with clear fluid arise. General symptoms, such as malaise and fever are also essential for the disease. Specific antitoxic antibodies can prevent syndrome development.

Staphylococcal ***peptidoglycan*** also possesses superantigenic activity. It stimulates inflammation and promotes chemotaxis of host leukocytes (***endotoxin-like*** activity).

Protein A is anchored within the cell wall of most of *S. aureus* strains. It binds to Fc portion of IgG molecules of different mammalian species including humans. Protein A is considered to hinder the complement activation and IgG binding to the immune cells.

Capsule of *S. aureus* supports microbial survival within phagocytes.

Besides exo- and endotoxin production, staphylococci can elaborate a large number of destructive enzymes.

S. aureus ***coagulase*** is capable of converting serum prothrombin into thrombin that activates blood coagulation with fibrin clotting. Fibrin threads on the microbial surface allow staphylococci to avoid phagocyte attachment.

Staphylokinase activates plasminogen thereby promoting fibrinolysis of blood clot within 24-48 hours.

The staphylococci produce ***hyaluronidase***, or spreading factor that breaks down hyaluronic acid of connective tissue that facilitates microbial invasion.

Lecithinase of *S. aureus* hydrolyzes the lecithin – phospholipid component of cellular membranes.

Staphylococcal ***β-lactamases*** break down the bonds within the beta-lactam ring causing microbial insusceptibility to beta-lactam antibiotics. Only specially designed beta-lactam drugs (e.g. methicillin, oxacillin, several cephalosporins and carbapenems) overcome beta-lactamase action.

β-Lactamase production is usually under the plasmid control. Nevertheless, strains of ***methicillin resistant Staphylococcus aureus*** (or ***MRSA***) have been appeared from chromosome-dependent alteration of ***penicillin-binding proteins*** (***PBP***). The bacteria produce modified protein ***PBP2a*** with low affinity to beta-lactam antibiotics. It is encoded by chromosomal gene ***mecA***.

It was found later that staphylococcal resistance to methicillin confers microbial insusceptibility for almost all of beta-lactams. Last decades MRSA has become a tremendous problem for public health as they provoke numerous life-threatening infections non-responsive to antibiotic therapy.

Resistance

Staphylococci are relatively resistant bacteria. They can propagate in 10% sodium chloride medium. These microbes develop resistance to drying, freezing, heating (maintain their viability for more than 1 hour at 70°) and some chemical substances. Boiling rapidly inactivates microbial cells. Also staphylococci are sensitive to chlorine-containing disinfectants and certain aniline dyes.

Pathogenesis and Clinical Findings of Staphylococcal Infections

Large number of mammalian species including humans suffers from staphylococcal infections.

Nevertheless, staphylococci, especially *S. epidermidis* and *S. saprophyticus*, are the representatives of the normal flora of human skin and respiratory tract. Nasal carriage of *S. aureus* is revealed in 40-50% of humans. But great number of pathogenicity factors, including toxins and destructive enzymes, and considerable invasive capacity ensure staphylococcal virulence.

Staphylococci, predominantly *S. aureus*, cause ***local*** and ***generalized*** (i.e, ***invasive***) ***infections***.

Staphylococci enter the host through the skin and mucous tissues that is followed by local microbial propagation. Finally, they can overcome tissue barriers and infect the blood.

Staphylococcus aureus can cause or participate in ***suppurative*** local lesions of all body tissues – furuncles (***boils***), carbuncles, paronychia, hidradenitis, chronic pyoderma, ***abscesses*** and ***phlegmons***, periostitis, ***osteomyelitis***, otitis, appendicitis, cholecystitis, pyelonephritis and many other diseases.

Also it causes ***pneumonia***, peritonitis and ***meningitis***, as well as ***post-operative wound infections***. Almost all of these situations can progress towards disseminative infection resulting in ***staphylococcal septicemia***.

S. aureus takes an active participation in ***mixed infections***.

Actions of numerous toxins produce clinical manifestations of *specific* staphylococcal infections. They should be often regarded as *toxic infections*.

Staphylococcal ***food poisoning*** appears after ingestion of foodstuffs (diary products, cakes, pastry, ice cream, etc.) contaminated with pathogenic bacteria. Enterotoxins are thermostable and withstand heating at 100^{o}C for 30 min.

Scalded skin syndrome and ***toxic shock syndrome*** result from infections, caused by particular toxin-producing strains of staphylococci.

Anti-toxic antibodies that appear in staphylococcal toxic infections can neutralize toxin action. Nevertheless, the majority of staphylococcal infections are shown to trigger only low-grade immune responses of short duration. Phagocytosis is considered to be the substantial mechanism for staphylococci elimination.

S. epidermidis is generally less pathogenic, than *S. aureus*, but it is emphasized to cause highly deleterious complications in immunocompromised patients and in patients with implanted prosthetic devices (e.g. bacterial endocarditis and septicemia).

Overall, *S. aureus* and *S. epidermidis* are from the most common causative agents of ***hospital-acquired*** infections.

S. saprophyticus can affect the urogenital tract of young women and may be the rare cause of wound infections.

Laboratory Diagnosis of Staphylococcal Infections

Specimens are obtained from pus, wound discharge, tracheal aspirate, spinal fluid, sputum, urine, blood, contaminated foodstuffs, lavage fluids, feces, etc.

Microscopy is used as preliminary test to validate staphylococcal infection. Gram-stained smear examination usually reveals gram-positive cocci arranged into grape-like clusters or settled separately.

Rapid differential diagnosis of various staphylococcal species directly in clinical samples can be elaborated with molecular ***genetic*** tests, e.g. ***PCR***.

For ***microbial culture isolation*** specimens are planted on blood agar and egg yolk salt agar. The latter medium is selective for staphylococci. In cases of septicemia blood is inoculated into glucose broth.

S. aureus culture renders hemolysis on blood agar. Also it produces golden pigment and positive lecithinase activity in yolk salt agar.

Catalase test allows to discern staphylococci and streptococci (the latter are devoid of catalase activity).

Positive coagulase test is essential for *S. aureus*. The identification is performed by inoculation of microbial culture into citrated rabbit plasma. If clot forms within hours, the test is ascertained to be positive.

Also *S. aureus* but not other staphylococci ferment mannitol and produce thermostable nuclease. These tests may be valuable for *S. aureus* discrimination.

Serological tests are of limited value in verifying of staphylococcal infection.

Susceptibility testing finalizes the investigation for staphylococci. Disk diffusion and broth microdilution methods are used.

Methicillin-resistant *S. aureus* (i.e., ***MRSA*** strains) are determined by ***PCR***.

Treatment and Prophylaxis of Staphylococcal Infections

Drugs that block cell wall synthesis are most suitable for staphylococcal infection ***treatment***. Unfortunately, most of *S. aureus* isolates produce beta-lactamases, thus conferring resistance to penicillin G or amoxycillin. Therefore, beta-lactamase-resistant penicillins (e.g., oxacillin and ***methicillin***) and ***cephalosporins***, as well as carbapenems, are used here for antibacterial therapy. In combination with antibiotics, the specific inhibitors of β-lactamases (e.g., clavulanic acid) give additional beneficial effect for treatment outcome.

Resistance to oxacillin and methicillin appears in about 20% of *S. aureus* (i.e., ***MRSA strains***) and approximately 75% of *S. epidermidis* strains. For treatment of these bacteria other inhibitors of cell wall synthesis – glycopeptides ***vancomycin*** or teicoplanin – are used. They should be administered in combination with antibiotics, blocking microbial protein synthesis – aminoglycosides, macrolides, lincosamides, tetracyclines (e.g., ***tigecycline***), and ***linezolid***.

In cases of chronic staphylococcal infections especially in immunocompromised patients and infants the specific passive immune therapy can be administered (e.g., anti-staphylococcal γ-globulin).

For activation of anti-staphylococcal immunity the toxoid, derived from *S. aureus* alpha-toxin can be administered as well. Specific prophylaxis with staphylococcal toxoid is recommended for patients, supposed to be susceptible to staphylococcal infections.

Adequate hospital disinfection and prevention of staphylococcal carriage among medical personnel can restrict the spread of staphylococcal infections.

PATHOGENIC STREPTOCOCCI

The History of Discovery

T. Billroth described first streptococci in patients with wound infections in 1874. L. Pasteur discovered streptococci in patients with sepsis in 1880; F. Fehleisen in 1883 and F. Rosenbach in 1884 isolated the pure culture of these bacteria.

Classification of Streptococci

Streptococci belong to the family *Streptococcaceae* and genus *Streptococcus.* Not long ago the family contained one more genus *Enterococcus*. Later it was placed into the separate family *Enterococcaceae*.

Streptococcus genus comprises more than 60 species.

The main microbial species that cause pathology in humans are: ***S. pyogenes*** and *S. agalactiae*; oral streptococci ***S. mutans*** and *S. sobrinus* (causative agents of caries); causative agent of pneumonia ***S. pneumonia*** or pneumococcus.

Opportunistic pathogens ***E. fecalis*** and *E. faecies* are the main representatives of genus *Enterococcus*.

Historically all streptococci were divided according to their hemolytic activity into ***α-hemolytic*** (produce green zones of hemolysis, e.g. ***viridans streptococci*** like *S. mutans*), ***β-hemolytic*** that develop clear zones of

complete hemolysis, e.g. *S. pyogenes* and *S. agalactiae*, and ***non-hemolytic*** streptococci without hemolysis.

Also streptococci are classified by their antigenic properties into serogroups (A-U), some groups are further divided into types.

Serological group division proposed by R. Lancefield is based on polysaccharide cell wall antigens.

S. pyogenes pertains to group A, *S. agalactiae* is the member of group B. Oral streptococcus *S. sanguinis* is related with group H.

Enterococci *E. fecalis* and *E. faecies* belong to group D.

Streptococci of A group are further divided into more than 80 serotypes due to the structural differences of their M protein antigen.

S. pneumoniae and viridans streptococci (e.g., numerous oral streptococci *S. mutans*, *S. salivarius*, *S. mitis* and others) are beyond of Lancefield classification. *S. pneumoniae* is subdivided into more than 90 serotypes on the basis of specific capsular carbohydrate antigens.

Structure and Properties of Streptococci

Morphology

Streptococci are ***gram-positive*** spherical microbes, 0.5-1 μm in diameter, which are usually clustered into ***chains*** or pairs. They are non-motile bacteria without flagella and spores. The cells possess pili, containing M protein and lipoteichoic acid.

Pneumococci are ***paired cocci*** of lancet-like shape.

Enterococci are the motile bacteria that carry one polar flagellum.

Many streptococcal species of A, B and C groups as well as pneumococci, produce the capsule. It is composed predominantly of hyaluronic acid.

Cultivation

Streptococci are relatively fastidious bacteria. The temperature range for their growth is rather narrow within the limits of 20-40°. They are cultivated on blood, serum or sugar agar and broth, pH 7.2-7.4. On solid media streptococci develop small, gray, translucent colonies. The growth in the sugar broth appears as fine precipitate near the walls and bottom of the test tubes.

Many strains are hemolytic (see above). Green hemolysis zone results from conversion of hemoglobin into methemoglobin (viridans streptococci and pneumococci).

Biochemical properties

Streptococci are facultatively anaerobic or aerotolerant microorganisms. These bacteria ferment carbohydrates (e.g. glucose, maltose, lactose, sucrose, etc.) with acid formation without gas. They are lack of proteolytic activity, can't liquefy gelatin and don't reduce nitrates into nitrites.

Streptococci are catalase-negative bacteria, whereas entrococci reveal minor catalase activity. Also enterococci easily grow in the presence of bile and 6.5% NaCl, hydrolyze esculin that distinguishes them from streptococci.

Streptococci produce great variety of invasive and toxic enzymes (see below).

Antigenic structure

Streptococci possess a great number of antigenic determinants within the cell wall and capsule, which are of oligosaccharide and protein nature.

Group-specific polysaccharide antigens of the cell wall are thermostable and contain different side residues of amine sugars and teichoic acids.

M protein of group A *S. pyogenes* is presented in more than 80 structural variations. It is heat- and acid-labile substance.

T protein and *R protein* present some other streptococcal surface antigens. They can be used for further differentiation of streptococci.

P substance is the nucleoprotein fraction, which is common in most hemolytic streptococci.

Virulence factors

Group A streptococci are able to produce a great number of toxic substances, agressins and invasion enzymes.

M protein is regarded as the most significant virulence factor of streptococci. M proteins are divided now into class I and class II molecules due to the reactions with different antibodies. The patients with rheumatic fever are usually infected with class I M protein streptococci.

M protein, which is encoded by *emm* gene, ***inhibits phagocytosis*** and promotes the adhesion of streptococci to the host tissues. This is the main mechanism of streptococcal virulence, since the lack of *emm* gene results in efficient phagocytosis of the invaded pathogen.

It was elucidated also that M protein binds to H factor of alternative pathway of complement activation as well as to host fibrinogen molecules. Both interactions suppress complement activation and, more important,

severely reduce the opsonization of streptococci, thus inhibiting phagocytosis. Likewise, M protein is shown to activate bradykinin, stimulating tissue inflammation.

Adhesive capacity of M protein facilitates streptococcal entry into infected cells that results in intracellular persistence of bacteria.

Hyaluronic acid capsule is also required to withstand phagocytosis.

Streptococcal pyrogenic exotoxins A and ***C*** (or ***erythrogenic toxins***) and ***streptococcal mitogens*** work as ***superantigens***, resembling staphylococcal enterotoxins. They trigger endotoxic shock with pyrogenic reactions that ensues from the massive release of proinflammatory cytokines (IL-1, IL-6, gamma interferon, α-TNF, etc.)

Streptococcal pyrogenic exotoxin B or ***streptococcal proteinase*** is an extracellular cysteine protease produced by all group A streptococci. It can directly activate IL-1 via specific intramolecular proteolysis as well as host tissue metalloproteases, enhancing inflammation and bacterial invasion.

Streptococcal pyrogenic exotoxins A and C are encoded by genes of a lysogenic temperate bacteriophage, while exotoxin B is of chromosomal origin.

Streptococcal adhesins comprise great variety of virulence factors that ensure streptococcal adherence and intracellular penetration. Among them are above mentioned M protein, ***lipoteichoic acid***, ***fibronectin-binding protein***, ***collagen-binding protein*** and many others.

Streptococci express different ***IgG-*** and ***IgA-binding proteins***. These proteins prevent antibody-mediated opsonization and deregulate mucous tissue immunity.

The group of ***plasminogen-binding proteins*** contains several factors, including the enzyme ***streptokinase***. They convert plasminogen into plasmin on the bacterial surface. Plasmin, attached to the microbial cells, activates extracellular metalloproteases or collagenases forwarding tissiue damage and enhancing invasion.

Many other enzymes are produced by virulent streptococci.

Streptococcal ***hyaluronidase*** destroys hyaluronic acid of connective tissue facilitating microbial invasion.

C5a peptidase of streptococci splits C5a complement fragment, preventing efficient chemotaxis of phagocytes.

Streptococcal ***streptodornase*** or ***deoxyribonuclease*** hydrolyzes host DNA.

Different types of hemolysins are revealed in streptococci. *S. pyogenes* produces two main hemolysins (***streptolysins***): ***streptolysin O*** and ***streptolysin S***.

Streptolysin O is a protein that contains free -SH groups, being sensitive to the oxygen. It induces high-titer synthesis of specific antibodies during infection.

Streptolysin S is of peptide nature, causing the hemolytic damage of cellular membranes. Also it may trigger apoptosis of infected cells and stimulate inflammatory response.

Resistance

Streptococci are not highly resistant bacteria, but they can withstand low temperatures, and survive for months in pus and sputum. They are killed at temperature of 70°C within one hour. Conventional disinfectants readily destroy them (e.g., phenol in concentrations of 3-5% inactivates bacteria in 15 minutes).

Pathogenesis and Clinical Findings in Streptococcal Infections

Group A representative ***Streptococcus pyogenes*** is the major streptococcal pathogen. It affects almost any body tissue or organ, thereby causing great variety of pyogenic local and invasive infections.

According to WHO data, group A streptococci account for at least 500,000 patients' death cases annually.

Among local infections are streptococcal ***pharyngitis*** or ***sore throat***, streptococcal ***pyoderma***, ***erysipelas***, ***cellulitis***, ***wound infections*** and some others. These disorders can be followed by serious complications, such as streptococcal pneumonia, meningitis, infectious acute and subacute endocarditis with possible fatal outcome.

Toxic and invasive streptococcal infections involve ***scarlet fever***, ***necrotizing fasciitis***, ***puerperal fever***, ***streptococcal toxic shock syndrome*** and ***septicemia***.

Non-suppurative ***sequelae*** *of streptococcal infections* include ***post-streptococcal acute glomerulonephritis*** and ***rheumatic fever***.

The infections are transmitted by ***air droplet route***, by direct contact, through skin lesions, etc.

Streptococcal sore throat is the most frequent infection caused by β-hemolytic streptococci. Bacteria attach to pharyngeal epithelium via the number of adhesins. The disease is characterized by throat pain, fever, nasopharyngitis, tonsillitis with purulent exudates, enlargement of cervical lymph nodes, etc.

Erysipelas is the specific streptococcal skin infection. Group A strains enter the skin through various lesions and penetrate the epidermis. The disease reproduces typical skin inflammatory damage with erythematous superficial skin layers.

Necrotizing fasciitis is a severe painful streptococcal disorder that affects subcutaneous tissues and fascia. It shows evident tendency to rapid spread into underlying tissues resulting in their necrosis and gangrene.

Scarlet fever is caused by group A streptococci that produce streptococcal pyrogenic exotoxins A, B and C. The symptoms result from systemic toxin action. They involve fever, generalized rash, bright "strawberry" tongue, skin desquamation. The disease profoundly affects cardiovascular system especially microcirculation.

Streptococcal toxic shock syndrome is the highest manifestation of toxigenic streptococcal infections. Hyperproduction of toxins and mitogens with superantigenic activity leads to hypotension and deep multiple organ failure that may cause patient's death. Beta-hemolytic streptococci of M protein types 1, 3, 11, 12, 28 are predominantly associated with shock appearance.

Poststreptococcal acute glomerulonephritis is a typical immune complex disease that evolves 2-3 weeks after streptococcal infection. The nephritogenicity of group A streptococci is related to particular M protein serotypes of *S. pyogenes*, such us ***M 12*** (predominant), 1, 2, 4, 49, 56, 57, and 60. Several immune mechanisms take part in disease pathogenesis. Among them are deposition of immune complexes on glomerular basal membrane, that is followed by complement activation, the production of antibodies, cross-reactive with streptococcal and glomerular antigens, direct damage of glomeruli by streptococcal enzymes and toxins. It was shown that renal glomerular membrane shares antigen epitopes with streptococcal M12 protein. This "antigen mimicry" provokes autoimmune reactions.

Rheumatic fever is a most serious delayed sequel of previous streptococcal infection. It arises within 1-5 weeks after group A streptococcal pharingitis (sore throat) or scarlet fever.

Rheumatic fever affects predominantly children or young persons. It is characterized by fever, mild polyarthritis without deformations, cardiovascular disorders that include heart inflammation (*endomyocarditis* and *pericarditis*) with systemic vasculitis, CNS involvement (*chorea*), skin manifestations (*erythema marginatum*).

Without adequate treatment the carditis leads to valves damage with *chronic valvular heart disease* progression. Finally the *chronic heart failure* can develop.

Rheumatic fever is the intermittent disease. Every secondary attack enhances valvular injury.

The disease is proven to be of ***autoimmune origin***. M protein is accounted as a major streptococcal antigen that renders antigenic mimicry with host cardiac and skeletal myosin, tropomyosin, laminin, keratin and other substances. Thus, rheumatic fever is provoked and supported by autoreactive antibodies and T cells that cross-react with streptococcal antigens and cardiac tissues. Subsequent immune complex deposition induces complement activation. These autoimmune mechanisms lead to profound host tissue lesions.

In certain clinical conditions some other representatives of *Streptococcaceae* family can cause the diseases in humans.

For instance, group B ***Streptococcus agalactiae*** elicit ***neonatal meningitis*** and sepsis of newborns and infants. These bacteria colonize vaginal mucosa of 10-30% of healthy women. The newborn becomes infected during delivery and may develop severe meningitis with lethality of 30-50%.

S. mutans as well as *S. sobrinus* takes part in dental plaque formation by synthesis of long-chain polysaccharides from sucrose thereby promoting ***caries*** initiation. The next progress of caries is related with fermentation of food-derived "table sugars" by *S. mutans*. It results in accumulation of lactic acid and tooth enamel decalcification with formation of caries lesion.

S. mitis and other viridans streptococci can cause the individual cases of ***bacterial endocarditis***.

Enterococci belong to the part of normal enteric microflora; nevertheless, they cause urinary tract infections. Being highly resistant to antimicrobial agents, they may cause severe hospital-acquired ***opportunistic*** infections in immunocompromised persons.

Streptococcus pneumoniae (or ***pneumococcus***) is the major causative agent of ***community-acquired pneumonia*** in groups of all ages. According to WHO data, pneumococcal pneumonia leads to more than 1 mln death cases annually in children before the age of five.

The disease severity is related with multiple virulence factors of pnemococci – polysaccharide ***capsule*** that protects bacteria from phagocytosis and opsonization; membrane-affecting exotoxin ***pneumolysin***; pneumococcal ***C-substance*** from cell wall teichoic acids that

activates complement system and triggers host inflammatory response; ***IgA proteases*** suppressing mucosal immunity.

Besides community-acquired pneumonia, *S. pneumoniae* plays the substantial role in etiology of ***sinusitis***, ***acute otitis media*** (about 40% of total cases), and ***bacterial meningitis*** in adults. It is generally ascertained that pneumococcal meningitis demonstrates extremely severe manifestations with mortality rate from 15 to 60%.

Post-streptococcal immunity is usually type-specific. Thus, it doesn't prevent the reinfection with another type of bacteria. The immunity is of a moderate grade and duration. Antibodies and immune T cells are directed to all major streptococcal antigens. Hypersensitivity reactions are shown to be common in most of streptococcal infections.

Laboratory Diagnosis of Streptococcal Infections

Specimens are obtained from the site of streptococcal infection. A throat swab, pus, wound discharge, blood, urine are examined.

Microscopy of specimens that reveals gram-positive single or short chain cocci is an auxiliary test, since the viridans streptococci may be found in clinical material as normal microflora.

Group A bacteria can be rapidly identified by immunofluorescence.

For cultivation the specimens are planted on blood agar and sugar broth. The primary growth appears in 1-2 days. Blood cultures are controlled within 5-7 days or even more.

The character of blood agar hemolysis is evaluated. Group A streptococci produce beta-hemolytic colonies.

Streptococci are catalase negative.

For definitive identification serologic grouping and typing of streptococci according to Lancefield classification is made by slide agglutination and precipitation tests.

Specific carbohydrate streptococcal antigens can be determined also by ELISA tests.

S. pyogenes is the single streptococcal representative rendering positive ***PYR-test*** (hydrolysis of pyrrolidonyl-β-naphthylamide substrate). In addition, *S. pyogenes* is sensitive to antibiotic bacitracin.

Serological diagnosis of group A streptococcal infections estimates the titer rise of antibodies to streptolysin O (basic test), streptokinase, hyaluronidase and DNAse (auxiliary tests). High titers of antistreptolysins

(> 250 units) appear mainly in rheumatic fever patients indicating recent or relapsing infection.

S. agalactiae is identified by so-called ***CAMP test*** (according to R. Christie, N.E. Atkins, and E. Munch-Peterson, who proposed this method). The test includes co-cultivation of *Streptococcus agalactiae* with *S. aureus* on blood agar. Usually two-streak plating of *S. agalactiae* is performed perpendicular to one-streak inoculation of hemolytic staphylococci. As a result, butterfly-like hemolysis enhancement of *S. agalactiae* appears.

Unlike conventional streptococci, ***enterococci*** easily grow in presence of bile and 6.5% NaCl. They can hydrolyze *esculin* that discriminates them from other streptococci.

S. pneumonia or ***pneumococci*** are gram-positive lancet-shaped diplococci. They develop alpha-hemolysis on blood agar. Their growth is inhibited by anti-microbial agent *optochin*. Also pneumococci are readily lysed in bile-containing media.

S. pneumonia is typed by capsular polysaccharide antigen into more than 90 serovars.

For rapid pneumococcal identification the slide microscopical test of capsule swelling is used. The specimen is treated by polyvalent antiserum that results in swelling of polysaccharide microbial capsule.

Streptococcal ***susceptibility testing*** is performed by disk diffusion and broth dilution methods.

Treatment and Prophylaxis of Streptococcal Infections

Beta-hemolytic group A streptococci are sensitive to benzylpenicillin, macrolides and azalides. Early ***treatment*** of streptococcal infections with penicillin interrupts autoimmune response against streptococcal antigens thus preventing poststreptococcal glomerulonephritis and rheumatic fever.

Benzylpenicillin (*penicillin G*) or *ampicillin* remain the drugs of choice for treatment of pneumococcal diseases caused by fully sensitive pneumococcal isolates; but penicillin-resistant strains gradually arise.

Enterococcus spp. is extremely resistant to many antibiotics. The bacteria display intrinsic resistance to most of beta-lactams, including cephalosporins. Also they are resistant to sulfonamides (co-trimoxazole) and develop medium resistance to fluoroquinolones and aminoglycosides.

Combination of penicillinase-sensitive penicillins (benzylpenicillin, ampicillin) or vancomycin with aminoglycosides is regarded as the optimal therapy of enterococcal infections.

For specific prophylaxis of pneumococcal diseases in children and adults various kinds of pneumococcal vaccines are actively used now.

The most common are *pneumococcal conjugate vaccine* (PCV13) containing antigens of 13 bacterial types and *pneumococcal polysaccharide vaccine* (PPSV23) against 23 types of pneumococci. They successfully prevent the development of pneumococcal infections.

For prophylaxis of group A streptococcal infections an experimental chemical vaccine, containing various M proteins of group A streptococci is being worked out.

PATHOGENIC PSEUDOMONADS AND OTHER NONFERMENTING GRAM-NEGATIVE BACTERIA

Aerobic ***nonfermenting gram-negative bacteria*** comprise the group of pathogens that hold the leading positions as causative agents of human ***hospital-acquired*** (or ***nosocomial***) infections. These bacteria belong to the related microbial families *Pseudomonadacea, Xanthomonadaceae*, *Moraxellaceae*, and *Burkholderiaceae*.

Within this group the limited number of microbial species, namely ***Pseudomonas aeruginosa***, ***Acinetobacter baumannii***, and ***Stenotrophomonas maltophilia***, demonstrate the highest clinical relevance.

They provoke severe complications, suppurative and wound infections in patients of intensive care units, burn centers and surgery departments. Moreover, all of them demonstrate the ***extreme levels*** of ***resistance to antimicrobial agents***.

Pseudomonas aeruginosa is the most common life-threatening pathogen. Normally, it is a saprophytic microbe that can be foumd on human skin and mucosal tissues, but it causes serious outbreaks of nosocomial infections especially in patients with suppressed immunity.

The History of Discovery

Initial description of *Pseudomonas aeruginosa* was presented by French pharmacist Carle Gessard as far back as in 1882.

Similarly, first representatives of *Acinetobacter* genus were discovered by M.W. Beijerinck in 1911. Nevertheless, active study of multidrug-resistant acinetobacters, e.g., *Acinetobacter baumannii*, commenced only from early 1990s.

First type strain of *Stenotrophomonas maltophilia* was isolated in 1958 by R. Hugh.

Classification of Nonfermenting Gram-negative Bacteria

Pseudomonas aeruginosa pertains to the family *Pseudomonadacea*, genus *Pseudomonas*.

Stenotrophomonas maltophilia is the member of *Xanthomonadaceae* family.

Pathogenic species from *Acinetobacter* genus *A. baumannii* and *A. baylyi* belong to the family *Moraxellaceae*.

Finally, pathogenic representatives of *Burkholderiaceae* family *Burkholderia cepacia* cause hospital-acquired infections; zoonotic agents *B. mallei* cause *glanders*, and *B. pseudomallei – melioidosis*.

Structure and Properties of *Pseudomonas aeruginosa*

Morphology

Pseudomonas aeruginosa is the major pathogen from the group of nonfermenting gram-negative bacteria. Pseudomonads are small gram-negative rods measuring about 2 μm. They are single non-sporeforming motile bacteria with one polar flagellum. Multiple pili and fimbriae promote microbial attachment to epithelial cells.

Multiple mucoid strains typically isolated from patients with cystic fibrosis produce large amounts of alginate exopolysaccharide that enwrapps bacterial cells.

Cultivation

Pseudomonads grow well on basic nutrient media. *P. aeruginosa* can propagate at 42°C.

During cultivation *P. aeruginosa* display smooth or mucoid round greenish colonies. The color of colonies results from overproduction of non-fluorescent bluish pigment ***pyocyanin***; in lesser extent the bacteria produce fluorescent green pigment *pyoverdin*, ruby-colored pigment *pyorubin* or the black pigment *pyomelanin*.

All pseudomonads actively form ***biofilms*** on adjacent surfaces due to the extensive production of adhesive exopolysaccharides.

Certain *P. aeruginosa* isolates can cause hemolysis.

Selective media for culture of *P. aeruginosa* contain various substances (e.g., *cetrimide* or *acetamide*) that support selective growth of pseudomonads.

Biochemical properties

Pseudomonads are ***obligate aerobes***. They don't ferment but oxidize glucose. These bacteria produce oxidase and catalase.

P. aeruginosa liquefy gelatin and hydrolyze casein without formation of H_2S or indole, and reduce nitrates to nitrites.

Antigenic structure

Antigenic epitopes of pseudomonads are localized predominantly within lipopolysaccharides of the cell wall (group-specific somatic ***O-Ag***) and microbial flagellar proteins (type-specific ***H-Ag***).

Virulence factors

P. aeruginosa expresses the broad scope of virulence factors.

The bacteria possess the structures of ***type II***, ***III*** and ***VI secretion systems*** that deliver virulence proteins into affected cells.

Multiple ***adhesins*** promote tight microbial attachment to the cells and tisuues.

Bacterial ***exopolysaccharides*** protect bacteria from phagocytosis and create the ground for ***biofilm*** formation.

Cell wall lipopolysaccharides possess ***endotoxin activity***.

P. aeruginosa synthesize ***exotoxin A***, which blocks protein synthesis by ribosylation of cellular elongation factor 2 (EF-2).

Exotoxins *ExoU* (phospholipase), *ExoY* (adenylate cyclase), *ExoS* and *ExoT* (ribosyltransferases) inhibit separation of cells after their division (***cytokinesis***) thereby grossly hampering the wound healing.

Hemolysins (*phospholipase C* and *lipase*) directly damage cell membranes.

The variety of agressive ***exo-enzymes*** (*collagenase*, *elastase*, proteases) destroys the components of connective tissue and intercellular tight junctions. *Neuraminidase* hydrolyzes host sialic acids.

Microbial ***siderophores*** provide iron supply for bacterial cells.

Most of pseudomonads produce bacteriocins (***pyocins***).

Finally, *P. aeruginosa* has remarkable and highly versatile ***mechanisms of natural multidrug resistance*** to antibiotics, antiseptics and disinfectants.

For instance, primary mechanism of resistance rests on extremely poor permeability of bacterial LPS for β-lactam antibiotics. Their transport across the cell wall is possible only through the porin channels within bacterial envelope. Frequent ***mutations of porin proteins*** lead to blockade of β-lactam entry into microbial cells.

Furthermore, *P. aeruginosa* maintain extensive ***reverse transport*** (or ***efflux***) of antimicrobial agents outside to the microbial cell. At least 4 separate systems of efflux provide active backward transportation of multiple antibiotic classes – β-lactams, fluoroquinolones, aminoglycosides, and tetracyclines.

In addition, *P. aeruginosa* express ***β-lactamase*** enzymes encoded by chromosomal and plasmid genes. Among them there are ***metallo-β-lactamases*** that confer resistance to all β-lactams including carbapenems.

Resistance

P. aeruginosa reveals substantial resistance in the environment. It stays viable in tap water for 2.5 months, in distilled water – up to 1 year, in home dust – for several days. *P. aeruginosa* can survive even in diluted disinfectants such as quaternary ammonium compounds as the bacterium is resistant to many antibiotics and antiseptics. Nevertheless, it remains sensitive to chlorine-contaning biocides and 2% phenol solution. Similarly, the cells of *P. aeruginosa* easily lose their viability under the routine sterilization by heating or autoclaving.

Pathogenesis and Clinical Findings of *P. aeruginosa* Infections

Pseudomonads are widely distributed in nature. They inhabit soil, water, and colonize plants and animals. As the external environment plays

a substantial role in spread of *P. aeruginosa*, the infections caused by these bacteria are referred to as ***sapronoses***.

Despite the presence of potent virulence factors, *P. aeruginosa* rarely cause infections in immunocompetent host. In addition, the bacteria ***need a pre-existing injury*** of skin and mucosal tissues for successful adherence and colonization like in patients with wounds, burns, traumas and other lesions. By production of ExoT toxin *P. aeruginosa* inhibits cytokinesis and cell proliferation, thus preventing the closure of wound edges and maintaining conditions for microbial propagation.

As the result, *P. aeruginosa* infections evolve only in patients with different injuries, implanted prosthetic devices, chronic surgical diseases and tumors with impaired local and systemic immunity, or after immunosuppression. These persons usually stay in hospital intensive care units and departments of surgery for a long time.

Hence, *P. aeruginosa* is a ***leading nosocomial pathogen*** covering about 15% of all hospital-acquired infections.

P. aeruginosa colonizes integument tissues and penetrates into the skin or mucous membranes that can initiate bacterial dissemination.

The most common ***sources*** of *P. aeruginosa*-associated nosocomial infections are the ***hospital microbial carriers*** (e.g., patients or medical personnel).

The major ***transmission routs*** – *airborne* (via contaminated aerosoles) or by ***direct contact***.

Bacteria cause a plethora of local and generalized infectious processes, including ***wound suppurative infections***, abscesses and phlegmons with blue-green purulent discharge, osteomyelitis, otitis, meningitis, urinary tract infections.

Severe ***disseminative infections*** result in ***sepsis*** with septic shock, hemorrhagic skin necrosis, disseminated intravascular coagulation, and adult respiratory distress syndrome.

P. aeruginosa is the main agent, causing so-called ***ventilator-associated pneumonia*** (***VAP***) – severe lung injury in patients receiving *mechanical lung ventilation*.

Systemic infections and VAP are characterized by high mortality rate in the range of 40-50%.

A special case of *P. aeruginosa* infection is observed in patients with ***cystic fibrosis*** – inherited autosomal recessive disorder associated with impaired mucociliary clearance. These patients are highly sensitive to *P. aeruginosa* demonstrating deep chronic course of disease with worse prognosis.

All these clinical cases are strongly aggravated by natural ***multidrug resistance*** of *P. aeruginosa* strains.

Laboratory Diagnosis of *P. aeruginosa* Infections

The ***specimens*** are obtained from wound discharge, pus, urine, blood, spinal fluid, sputum, etc.

Microscopy reveals single gram-negative rods.

Microbial ***culture isolation*** is performed on blood agar and selective media with antiseptics (cetrimide, acetamide, and others).

Identification is based on the morphology of colonies with the presence of characteristic pigments, biochemical and antigenic properties. *P. aeruginosa* is an oxidase-positive bacterium able to grow at 42°C. Differentiation from other pseudomonads is possible by biochemical tests, serotype determination and pyocin typing (see table 1).

Table 1
Basic differential tests for pseudomonads

Test	**Species**		
	P. aeruginosa	*P. fluorescens*	*P. putida*
Cytochrome oxidase	+	+	+
Pyocyanin pigment	+	–	–
Fluorescence	+	+	+
Glucose oxidation	+	+	+
Acetamide culture	+	–	+
Growth at 5^0C	–	+	–
Growth at 42^0C	+	–	–
Gelatinase	+	+	–

For ***epidemiological purposes*** molecular ***genetic typing*** of *P. aeruginosa* isolates is conducted by various ***PCR***-based tests.

Treatment and Prophylaxis of *P. aeruginosa* Infections

The treatment of infections caused by *P. aeruginosa* is extremely difficult clinical condition that ensues from multidrug-resistant nature of these agents. They show the resistance actually to the most efficient antimicrobials (e.g., carbapenems in more than 50-60% of cases) retaining sensitivity only to polymyxin group members.

As standard treatment regimen carbapenems are applied in combination with respiratory fluoroquinolones (levofloxacin). Also anti-pseudomonad cephalosporins (cefepime) and aminoglycosides (amikacin) can be administered.

Multiresistant strains of *P. aeruginosa* are cured with ***polymyxin E*** (or ***colistin***) – about 95% of strains remain sensitive.

Specific treatment with anti-pseudomonad immunoglobulin is possible. For specific immunization inactivated polyvalent pseudomonad vaccine can be used with uncertain results. Specific prophylaxis is recommended for high-risk patients and in case of *P. aeruginosa* infection outbreaks.

Other Representatives of Nonfermenting Gram-negative Bacteria – *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*

Acinetobacter baumannii is the next common nosocomial pathogen pertaining to the group of nonfermenting gram-negative bacteria.

Since 1970s the number of multidrug-resistant strains of *A. baumannii* constantly arises. These bacteria are ubiquitous in nature being broadly found in soil and water. Also they are normal habitants of human skin.

The bacteria don't affect immunocompetent hosts and cause serious hospital-acquires infections only in ***immunocompromised individuals***. These pathogens become associated with ventilator-associated pneumonia in patients receiving artificial lung ventilation. Also they cause nosocomial endocarditis, meningitis, peritonitis, urinary tract infections and septicemia.

The hallmark of hospital isolates of *A. baumannii* is their ***striking multiple resistance to antimicrobial agents***. It is encoded by the number of pathogenicity islands with transposable genetic elements (integrons and transposons). The bacteria actively exploit the basic mechanisms of drug resistance – poor permeability of bacterial envelope, alterations of ribosomal structure, intensive ***drug efflux*** and synthesis of ***highly active β-lactamases*** including extended-spectrum beta-lactamases (***ESBL***) and metallo-β-lactamases.

As the result, *A. baumannii* renders multidrug resistance to β-lactams, aminoglycosides, fluoroquinolones, tetracyclines and co-trimoxazole.

Carbapenems are the current drugs of choice for treatment of *A. baumannii* infections as more than 95% of strains remain sensitive to them.

Similarly, *Stenotrophomonas maltophilia* is one more nosocomial pathogen of considerable medical notoriety that develops outstanding ***multidrug resistance***.

These microorganisms are also ubiquitous in nature being present in soil, water, various foodstuffs. They may colonize human nasopharingeal cavity and intestine.

S. maltophilia are intrinsically resistant to β-lactams including carbapenems, aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol. The mechanisms of resistance are generally similar to above described pathogens.

Like acinetobacters, stenotrophomonads affect immunocompromised individuals resulting in ***severe opportunistic infections***. High-risk group for infection comprises the patients of intensive care units, premature infants, cancer patients, HIV-infected persons in stage of AIDS and others.

The bacteria are transmitted mainly by ***airborne route***. They cause ***hospital-acquired pneumonia*** and systemic infection (***sepsis***) with high mortality rate. They can provoke also catheter-associated infections, microbial endocarditis, peritonitis, wound infections.

The treatment of *S. maltophilia*-associated disorders is performed with ***co-trimoxazole***, combined β-lactams (e.g. ticarcillin/clavulanic acid) and selected fluoroquinolones (levofloxacin or moxifloxacin).

Chapter 2

CAUSATIVE AGENTS OF SUPPURATIVE, WOUND AND HOSPITAL-ACQUIRED ANAEROBIC INFECTIONS

PATHOGENIC CLOSTRIDIA – CAUSATIVE AGENTS OF GAS GANGRENE

Gas gangrene is the severe polymicrobial wound infection. It is caused by various clostridial anaerobic microflora in association with pathogenic facultatively anaerobic bacteria (staphylococci, streptococci, gram-negative rods, etc.)

The History of Discovery

The major causative agent of gas gangrene *Clostridium perfringens* was first isolated by W. Welch and G. Nuttall in 1892.

About 15 years earlier in 1877 the first member of gas gangrene group *Clostridium septicum* was discovered by L. Pasteur and J. Joubert. Then R. Koch confirmed its ability to cause gas gangrene with edema.

In 1894 F. Novy described another clostridium species, which was later named as *Clostridium novyi*.

Finally *Clostridium hystolyticum* was identificated in 1916 by M. Weinberg and P. Seguin.

Classification of Pathogenic Clostridia

Clostridia of gas gangrene belong to the family *Clostridiaceae*, genus *Clostridium*. Among the disease causative agents are the species *Clostridium perfringens*, *C. novyi*, *C. septicum*, *C. hystolyticum* as well as *C. sordelli*, *C. fallax*, *C. ramosum* and some others.

Structure and Properties of Clostridia

Morphology

C. perfringens is typically ***gram-positive*** thick non-motile rod with rounded ends. It possesses oval ***spore*** of central or subterminal localization and forms ***capsule*** within the infected host.

C. novyi are rod-shaped motile peritrichous bacteria with subterminal spores. It doesn't form capsule.

C. septicum are polymorphic non-capsulated rods that can develop long filamentous forms. Microorganisms carry central or subterminal spore and peritrichous flagella.

C. hystolyticum in morphology is similar with previous two representatives.

Cultivation

C. perfringens is found to be the most aerotolerant among all other clostridia. Similar to other anaerobic bacteria, it is cultured on *iron sulfite agar*, *Schaedler agar* and glucose blood agar in anaerobic jars, *Kitt-Tarozzi* and other anaerobic media.

C. perfringens is able to blacken iron sulfite agar within first 6-8 h of cultivation.

In Kitt-Tarozzi medium *C. perfringens* renders homogenous turbidity with gas production.

C. novyi, *C. septicum* and *C. hystolyticum* are strictly anaerobic bacteria. On glucose blood agar *C. novyi* form rough fringed colonies with hemolysis.

C. septicum is cultured readily in meat-peptone broth. The bacteria develop a film on glucose blood agar. In agar stab cultures the colonies look like balls of wool.

Biochemical properties

All clostridia are ***obligate anaerobes***.

C. perfringens ferments glucose, sucrose, lactose, starch, and many other sugars with large amounts of acid and gas end products. They liquefy gelatin, coagulate blood serum and milk resulting in sponge-like clot. These bacteria reduce nitrates to nitrites. They produce butyric and acetic acids and large amounts of gases CO_2, H_2, H_2S and others.

C. novyi ferments glucose, maltose and glycerol with acid and gas production. They also liquefy gelatin and coagulate milk with small flakes.

C. septicum slowly liquefies gelatin, and utilize proteins with appearance of hydrogen sulfide and ammonia. Several mono- or disaccharides are metabolized with acid and gas formation

C. hystolyticum doesn't ferment sugars but reveals substantial proteolytic activity.

Antigenic structure

Serologic differentiation of *C. perfringens* is based on the antigenic variations of microbial toxins. Six main serovars A, B, C, D, E, and F are known. Type A is further divided into many subtypes. Types A, C and D are pathogenic for humans; B, C, D, and E affect animals.

C. novyi comprises 4 antigenic variants A, B, C, and D, where A type is the predominant pathogenic variant for humans.

C. septicum can be distinguished into 6 serovars according to their exotoxins.

C. hystolyticum has 5 antigenic variants, depending on toxin structure differences.

Virulence factors

All clostridia produce extreme variety of virulence factors that predominantly display potent ***enzymatic activity***.

α-Toxin of *C. perfringens* or ***phospholipase C*** displays high ***lecithinase*** activity that damages cell membranes, enhances vascular permeability and develops necrotizing activity.

β-Toxin is a potent necrotizing substance; ε-toxin increases vascular permeability in the gastrointestinal tract.

θ-Toxin or ***perfringolysin O*** demonstrates polyfunctional hemolytic, dermonecrotizing and lethal properties.

Other minor toxins also possess enzymatic properties. For instance, κ-toxin acts as collagenase, μ-toxin – hyaluronidase, δ-toxin develops hemolytic activity.

In addition, *C. perfringens* expresses potent ***enterotoxin***.

C. novyi produces at least 8 distinct toxins with hemolytic, lecithinase, protease and hyaluronidase activities.

C. septicum has 4 major toxins: α-toxin with lethal, hemolytic and necrotizing activity, β-toxin with DNase activity, γ-toxin is hyaluronidase and δ-toxin is hemolysin.

C. hystolyticum expresses 5 toxins, among them are α-toxin with lethal and necrotizing activity, β-toxin with collagenase activity, γ-toxin with protease activity, δ-toxin with elastase activity, and ε-toxin renders hemolytic activity.

Resistance

The spores of *C. perfringens* withstand boiling for time period of 8 to 90 minutes. The vegetative forms are most susceptible to hydrogen

peroxide and phenol in concentrations commonly employed for disinfection.

Spores of *C. novyi* survive in natural surroundings for a period of 20-25 years without losing their virulence. Direct sunlight kills them in 24 hours, boiling destroys them in 10-15 minutes. Spores withstand exposure to a 3% solution of formaldehyde for 10 minutes.

Pathogenesis and Clinical Findings in Gas Gangrene

Clostridia stay in the intestine of animals and humans (as ***sources of infection***) and discharge outside with feces. Spores of clostridia are constantly present in the soil. Hence, any contact with dust and soil particles inevitably results in contamination of skin and mucosal tissues with clostridial spores.

Gas gangrene develops, when grossly damaged soft tissues (muscles, adipose or connective tissue) become infected with spores of *C. perfringens* and other clostridia; it occurs in severe trauma with tissue crash, after septic abortion, in case of war wounds, or other similar situations.

Therefore, clostridial infection is predominantly transmitted by ***contact route***.

The causative agents of anaerobic infections require certain conditions for their germination and overgrowth. The basic one is *the presence of dead or damaged tissues* resulting in ***low oxidation-reduction potential*** (***state of anaerobiosis***). Characteristic type of injury (deep narrow wounds or contaminated crashed tissues) as well as patient state of health predisposes to the emergence of gas gangrene (for instance, diabetes mellitus strongly impairs tissue oxygenation).

Progressive propagation of pathogenic anaerobes leads to further degradation of body tissues thus aggravating anaerobic conditions.

Active spread of infection ensures relatively short ***incubation period*** – from several hours up to 4-5 days.

Gas gangrene targets primarily ***muscles*** and ***adipose tissue*** as they harbor a lot of potential substrates for microbial toxic enzymes (e.g., glycogen or phospholipids). As the result, exotoxins of clostridia cause expanding tissue necrosis and melting. It is followed by accumulation of gases like CO_2 and H_2 in soft tissues that is detected as ***gas gangrene***.

Growing ***edema*** blocks local circulation, thereby enhancing anaerobic conditions and toxin production.

Edema is characteristic for the first phase of the infection, and gangrene of the soft tissues progresses in the second phase.

Microbial exotoxins generate both local and systemic devastating effects, being spread throughout the body. The products of tissue decay render additional toxicity against host tissues.

As the result of massive edema and tissue necrosis with gas formation, the skin over the affected limbs becomes pale, then reddish and cyanotic with extensive hemorrhages. Deep destructive changes in subcutaneous adipose tissue, muscles, and fascias require urgent surgical treatment and systemic antimicrobial and antitoxic therapy.

The immunity arisen in the course of anaerobic clostridial infections is maintained predominantly by antitoxic antibodies. They neutralize the activity of multiple microbial toxins. However, the immune response is non-protective being of low grade. Without complex intensive treatment it is impossible to prevent the rapid disease progression.

Besides gas gangrene development, *C. perfringens* may cause severe ***necrotizing enteritis*** followed by deep damage of small intestine. It ensues from the action of clostridial β-toxin with potent cytotoxic and necrotizing activity.

In addition, *C. perfringens* are not so rare agents of ***food poisonings*** (or ***food toxinfections***). These disorders are related with production of enterotoxins by clostridia.

Laboratory Diagnosis of Gas Gangrene

The specimens for examination comprise the pieces of necrotic tissues, tissue fluids and wound discharges, surgical stitch materials, dressings, etc.

As preliminary test, ***microscopical examination*** of wound discharge for *C. perfringens* or other clostridia is made on the ground of their typical morphological characteristics. Also *immunofluorescence microscopy* can be applied for direct identification of clostridia in clinical samples.

Culture isolation is elaborated in anaerobic conditions (e.g., in anaerobic jars). Identification of microbial species takes into account their growth on iron sulfite agar, fermentation of carbohydrates, gelatin liquefaction and other biochemical tests, microbial serological properties.

To confirm the diagnosis, ***experimental injection*** of mice with broth culture filtrates for ***exotoxin detection*** as well as antitoxin-toxin ***neutralization reactions*** are performed.

Rapid diagnostic test for detection of clostridial ***exototoxins*** in clinical samples are based on ***ELISA*** test or indirect hemagglutination assay with erythrocyte antitoxic diagnosticum.

Genetic typing of clostridia species is performed by ***PCR***.

Treatment and Prophylaxis of Gas Gangrene

Treatment of gas gangrene comprises the intensive surgical management of wounds and injuries with removal of affected tissues up to limb amputations, massive antibiotic chemotherapy against anaerobic infection (e.g., with β-lactams, aminoglycosides, and metronidazole), infusion and detoxification therapy, administration of polyvalent purified and concentrated antitoxin against *C. perfringens* and other clostridia.

Hyperbaric oxygen therapy, blood transfusions and administration of inhibitors of proteolytic enzymes are the additional supportive measures for gas gangrene treatment.

Prophylaxis of gas gangrene is non-specific. It primarily includes the protection of wounds and injuries from contamination and their adequate surgical treatment.

CLOSTRIDIUM TETANI – CAUSATIVE AGENT OF TETANUS

The History of Discovery

A causative agent of tetanus was described first by A. Nicolaier in 1884, and isolated the pure culture by S. Kitasato in 1889.

Classification

Tetanus causative agent pertains to the family *Clostridiaceae*, genus *Clostridium*, species *C. tetani*.

Structure and Properties of *C. tetani*

Morphology

Clostridium tetani is a thin gram-positive rod about 5 μm in length. It is a motile Peritrichous bacterium with round terminal spore. Spore-containing cells resemble ***drumsticks***.

Cultivation

The temperature range for optimal propagation of *C. tetani* is 15-45°C. Usually it grows on the blood or sugar agar, pH 7.0-8.0 at 37°C being cultured in anaerobic conditions within anaerobic jars. At the end of cultivation small smooth colonies surrounded by slight zones of hemolysis are observed. The view of colonies resembles dew drops.

When cultured within the deep agar stabs, fragile cloud-like colonies appear. Cultivation in Kitt-Tarozzi medium results in homogenous turbidity with gas production.

Biochemical properties

As any other clostridia, *C. tetani* is ***obligate anaerobe***. It demonstrates generally poor biochemical activity. Tetanus causative agent doesn't ferment carbohydrates. Nevertheless, these bacteria liquefy gelatin, coagulate milk, and reduce nitrates to nitrites.

Antigenic structure

Clostridium tetani is divided into 10 serotypes accroding to variations of flagellar H-antigen. Somatic O-antigen is group-specific. Microbial exotoxin has common antigenic properties in all *C. tetani*.

Virulence factors

C. tetani expresses highly poisonous exotoxin composed of two fractions: ***tetanospasmin*** that causes the contraction of skeletal muscles, and ***tetanolysin***, which produces hemolysis.

The lethal activity of tetanus exotoxin succumbs only to the action of the most potent botulotoxin – one mouse lethal dose of dry tetanus toxin is about 5 ng. The mortal dose for humans is near 2-2.5 ng per 1 kg of body weight.

Tetanospasmin is ***Zn-containing metal protease*** that destroys synaptic vesicle-associated protein and synaptobrevin in the synapses of ***inhibitory neurons*** of central nervous system resulting in generalized muscular spasms.

Tetanolysin is the membrane-damaging fraction of exotoxin with hemolytic and cardiotoxic properties. Also it affects medullar nuclei and the neurons of autonomic nervous system.

Resistance

Heating at 60-70°C inactivates vegetative forms of clostridia within 30 minutes. The bacteria are sensitive to conventional disinfectants.

The spores are extremely resistant and keep viability in soil and dust for many years. They can withstand boiling for more than 1 hour. Standard disinfectants, such us 5% phenol or 1% formaldehyde inactivate clostridia spores only after 5-10 h of exposure.

Pathogenesis and Clinical Findings in Tetanus

C. tetani is the normal inhabitant of human and animal gut. *Animals and humans* are the major ***sources of infection***. Spores of clostridia appear in the soil with feces and may stay there for years.

Microorganisms enter the body through the injured skin or mucous membranes via soil-contaminated wounds or skin lesions (***contact route*** of the disease transmission). More often the disease affects children or agriculture workers.

C. tetani multiply in the site of primary contact and release exotoxin. Toxin undergoes retroaxonal or perineural lymphatics transport and moves into the spinal cord. It binds to ganglioside receptors of neurons, penetrates into the synapses and blocks the release of ***inhibitory neuromediators*** (glycin and γ- aminobutyric acid).

Impairment of inhibitory signalling leads to uncontrolled stimulation of neuromuscular synapses of motor neurons that elicits tonic or myoclonic ***striated muscle contractions***.

At the primary site of pathogen penetration persistent tonic muscular cramps are observed. The symptoms arise in descending order. At first the disease affects jaw muscles with tonic spasms (*trismus*) and face muscles (*risus sardonicus*). Then the back muscles and limbs are involved. Generalized tetanus muscular spasm is known as *opisthotonus*.

The disease ***prognosis is very serious***. In case of delay of treatment the developed tetanus results in lethal outcome in 40-50% of cases.

Anti-toxic natural immunity *is very* ***weak*** and can't prevent next tetanus infection.

Laboratory Diagnosis of Tetanus

In most of cases the clinical findings of tetanus are enough evident to make correct diagnosis.

For laboratory confirmation of the diagnosis of tetanus the ***specimens*** of wound discharge, biopsy tissue samples, or stitch material can be examined.

Investigation of tetanus toxin in clinical samples is performed by ELISA test, or by indirect hemagglutination test with erythrocyte anti-tetanus diagnosticum, or by neutralization reaction in mice.

For ***culture isolation*** the specimens should be previously heated at 80°C for 20 min to inactivate non-sporeforming bacteria. Then they are inoculated into Kitt-Tarozzi medium or upon blood agar that is placed into anaerobic jar. After several days of incubation the grown colonies undergo microscopy. Toxin accumulation is evaluated by *experimental mice infection*. The diagnosis is confirmed by neutralization reaction with anti-tetanus antibodies.

Treatment and Prophylaxis of Tetanus

Urgent prophylaxis of the disease depends on the level of initial antitoxic immunity of affected person. Prophylaxis covers all patients with traumas, burns, animal bites, etc.

Previously vaccinated individuals are immunized with tetanus toxoid. Non-immune patients obtain tetanus toxoid and human antitoxic anti-tetanus immunoglobulin.

For treatment of developed tetanus the high doses of ***human antitoxic tetanus immunoglobulin*** or horse antitoxic serum are used. In addition, anticonvulsive drug therapy is administered (diplacine, aminazine, diazepam, etc.)

Organized ***active prophylaxis*** is performed by vaccination with ***tetanus toxoid***. The toxoid is an essential constituent of complex ***ADPT polyvaccine*** (*adsorbed diphtheria, pertussis, tetanus vaccine with aluminum hydroxide* as adjuvant) or of combined toxoid preparation ***ADT***. Tetanus is the completely preventable disease.

The vaccination starts and repeated thrice at the first year of life. Subsequent booster is injected in 9-12 months and then reproduced every 10 years.

CLOSTRIDIUM DIFFICILE – CAUSATIVE AGENT OF PSEUDOMEMBRANOUS COLITIS

The History of Discovery

Initial description of *Clostridium difficile* was given by I.C. Hall and E. O'Tool in 1935. Nonetheless, etiological role of *C. difficile* in the development of pseudomembranous colitis and antibiotic-associated diarrheas was established by J.G. Bartlett only in 1977.

Structure and Properties of *C. difficile*

Morphology

Clostridium difficile is a motile gram-positive rod with round subterminal spore. Some strains may carry thin capsule.

Cultivation

Similar to other clostridia, they grow on the blood agar in anaerobic jars resulting in round-shaped middle-size colonies without hemolysis.

A selective and differential medium for *C. difficile* culture is *cycloserine-cefoxitin fructose agar*. Antibiotics cycloserine and cefoxitin suppress the growth of concomitant microflora. After overnight incubation in anaerobic conditions the growth of yellow fructose-positive colonies is observed.

Biochemical properties

These clostridia are chemically active and ferment various carbohydrate substrates (e.g., glucose, fructose, mannitol and others). They are able to liquefy gelatin. Combined mannitol fermentation and gelatin liquefaction distinguishes *C. difficile* from other clostridia.

Antigenic structure

C. difficile possess somatic polysaccharide and protein flagellar antigens as well as produce exotoxins with marked antigenicity. All members of *C. difficile* species express ***species-specific antigen*** – enzyme *glutamate dehydrogenase*.

Virulence factors

The main virulence factors of *C. difficile* are ***exotoxins A*** and ***B*** with enzymatic ***glycosyltransferase*** activity. When entered into enterocytes, they perform glycosylation of regulatory ***G-proteins*** (also known as "small GTPases") thereby blocking their normal activities. This leads to disorganization of cellular metabolism, impairment of polymerization of actin cytoskeleton, resulting in cell rounding and final apoptosis. In addition, *C. difficile* exotoxins damage tight junctions between enterocytes.

Exotoxin A (or ***enterotoxin***) activates the apoptosis of enterocytes and stimulates the synthesis of proinflammatory cytokines within intestinal wall followed by its neutrophil infiltration. This increases the permeability of the intestine resulting in diarrhea and bowel inflammation.

Similarly, ***exotoxin B*** (or ***cytotoxin***) elicits apoptosis of enteric epithelial cells and impairs tight junctions between enterocytes that leads to progression of inflammatory diarrhea.

Hospital strains of *C. difficile* often display ***multidrug resistance*** to antibiotics.

Pathogenesis and Clinical Findings of Diseases

C. difficile spores are widely present in natural environment. They maintain viability for a long time in the soil, water, or upon various fomites.

Up to 3% of adult population are the carriers of *C. difficile*. However, most of these community-acquired clostridia are nontoxigenic. By contrast, about 20% of patients receiving antibiotic treatment at hospitals become the carriers of ***hospital-aquired toxigenic*** *C. difficile*.

Hospital strains of *C. difficile* demonstrate enhanced virulence and multi-resistance to antimicrobial agents.

In patients that stay at hospitals and receive antibiotic therapy *C. difficile* is found as etiological agent of 15-30% of all ***antibiotic-associated diarrheas***, and about 50-75% of all cases of ***antibiotic-associated colitis*** with its severest clinical form ***pseudomembranous colitis***.

In majority of cases ***the source*** of *C. difficile*-associated infections is ***medical personnel*** of hospitals. That's why the clinical disorders caused by *C. difficile* are generally ascertained as ***hospital-acquired infections***.

The spread of infection occurs via ***fecal-oral route***. The predisposing factors to the contraction of *C. difficile* infection is the treatment of patients

with ***broad-spectrum antibiotics*** (β-lactam drugs – amoxycillin, cephalosporins, lincosamides – clindamycin, and to less extent – fluoroquinolones).

Above indicated antimicrobial agents suppress normal microflora of gut and grossly deteriorate colonization resistance of intestinal epithelium. These conditions foster the intestinal adherence and propagation of hospital-acquired toxigenic *C. difficile*.

The severety of colitis varies strongly, depending on toxin activities.

The released microbial toxins exert direct cytotoxic effects on intestinal epithelial cells resulting in their apoptosis and stimulate the production of proinflammatory cytokines. This leads to inflammatory damage of intestinal wall with neutrophil infiltration, microhemorrhages and formation of *pseudomembranes* composed of fibrin, inflammatory and necrotic cells (***pseudomembranous colitis***).

Bowel inflammation associated with death of intestinal epithelium and impairment of microcirculation breaks down the normal reabsorption of water and electrolites resulting in ***diarrhea*** and ***intestinal hemorrhages***.

The disease is also manifested by fever and intoxication; in most severe cases pseudomembranous colitis leads to the perforation of intestinal wall with subsequent peritonitis that strongly worsens clinical outcome.

The ***immunity*** against *C. difficile* is weak and not stable; the recurrent infections are observed.

Laboratory Diagnosis of Infections, Caused by *C. difficile*

Antibiotic-associated colitis caused by *C. difficile* requires laboratory confurmation with isolation of infectious agent and assessment of its toxigenicity.

Patient's *stool* is examined as primary clinical ***specimen***.

Rapid method for indication of *C. difficile* in stool detects species-specific microbial ***antigen*** – enzyme *glutamate dehydrogenase* – by ***ELISA*** test.

Rapid tests that discover ***exotoxins*** of *C. difficile* in clinical samples are based on ***serological*** testing (***ELISA*** tests and latex agglutination) and methods of ***molecular genetic analysis*** for toxin-encoding genes (e.g., ***PCR*** tests).

The presence of exotoxins in stool filtrates can be determined also by *neutralization of toxin cytopathic effect* with specific antitoxic antibodies in laboratory cell cultures.

Isolation of *C. difficile* is performed on selective media (e.g., *cycloserine-cefoxitin fructose agar*, where the growth of yellow fructose-positive colonies is observed). The bacteria are further identified by their specific biochemical and antigenic properties as well as antibiotic resistance.

Toxigenicity of isolated culture is confirmed by serological (ELISA) and molecular genetic tests (PCR).

Treatment and Prophylaxis of Pseudomembranous Colitis

The ***treatment*** of pseudomembranous colitis presumes the immediate cessation of current antibiotic therapy and administration of antimicrobial agents efficient against *C. difficile*. The drug of the first line of treatment is metronidazole. Vancomycin can be administered as well.

In order to ameliorate the curative effects of antibiotics and to prevent *C. difficile* infection flare-ups the ***probiotic***-containing biological products are administered (the cultures of *Lactobacillus acidophilus, Saccharomyces boulardii* or other bacteria of certain clinical value). They entail antagonistic activity against *C. difficile* hospital strains and restore colonization resistance of intestinal epithelium.

PATHOGENIC GRAM-NEGATIVE NON-SPOREFORMING ANAEROBIC BACTERIA

Non-sporeforming anaerobic bacteria cause a great variety of bacterial infections. Usually they affect human tissues together with facultative anaerobic and aerobic microflora. Anaerobic bacteria dominate in several body compartments, primarily in the oral cavity and gastrointestinal tract but even the infections of respiratory tract are proven to be of polymicrobial origin, where anaerobes occur in more than 50% of cases.

Since most of non-sporeforming anaerobes are the normal representatives of human flora, they can induce infectious inflammation only in high concentrations, or within normally sterile body cavities and

compartments, in immunocompromised patients, etc. Nosocomial strains of these bacteria demonstrate enhanced virulence and multidrug antibiotic resistance.

Classification of Pathogenic Gram-negative Non-sporeforming Anaerobes

Most of human anaerobic non-sporeforming pathogens pertain to related microbial ***families*** (see Table 2):

Bacteroidaceae,
Porphyromonadaceae,
Prevotellaceae,
Fusobacteriaceae,
Desulphovibrionaceae
with their major ***genera*** and ***species***:

Bacteroides (major species are *B. fragilis*, *B. ovatus*, *B. vulgatus*, *B. thetaiotaomicron*);
Porphyromonas (species *P. gingivalis*, *P. endodontalis*),
Prevotella (species *P. intermedia*, *P. melaninogenica*, *P. heparinolytica*);
Tannerella (major species is *T. forsythia*);
Fusobacteria (species *F. nucleatum*, *F. necroforum*);
Bilophila (species *B. wadsworthia*).

Table 2
Major medically relevant representatives of non-sporeforming gram-negative anaerobic bacteria

Taxonomic ranks	**Normal body habitation and the role in pathology**
Family *Bacteroidaceae* Genus ***Bacteroides*** Species: *B. fragilis, B. vulgatus, B. galacturonicus, B. coagulans, B. ovatus, B. pectinophilus, B. pyogenes, B. splanchnicus, B. thetaiotaomicron, B. uniformis, B. urealyticus* and many others	These bacteria represent a substantial part of microbiota of large intestine, oral cavity, and genital tract. They are isolated in wound infections, tissue abscesses, intra-abdominal and pelvic infections, osteomyelitis, sepsis

Family *Fusobacteriaceae* Genus ***Fusobacterium*** Species *F. necroforum, F. nucleatum* and others	Fusobactria inhabit oral cavity, large intestine, and genital tract. They can be isolated from tissue abscesses, in cases of wound infections, endocarditis, sepsis, acute necrotizing ulcerative gingivitis
Family *Porphyromonadaceae* Genus ***Porphyromonas*** Species: *P. gingivalis, P. endodontalis, P. asaccharolytica, P. circumdentaria* and many others	Normal inhabitants of oral cavity, large intestine, vagina; commonly isolated in odontogenic infections and mixed infections after animal bites
Family *Porphyromonadaceae* Genus ***Tannerella*** Species: *T. forsythia*	Present in oral cavity; commonly isolated from dental pockets in cases of gingivitis and periodontitis
Family *Prevotellaceae* Genus ***Prevotella*** Species: *P. intermedia, P. melaninogenica, P. bivia, P. buccae, P. buccalis, P. corporis, P. dentalis, P. denticola, P. distens, P. heparinolytica, P. nigrescens, P. oralis, P. oris* and many others.	These bacteria inhabit oral cavity, large intestine, and genital tract; they can be isolated in various odontogenic infections (e.g., periodonitis), pelvic infections, tissue abscesses, and sepsis.
Family *Desulfovibrionaceae* Genus ***Bilophila*** Species: *B. wadsworthia* and others	These bacteria exist in large intestine, oral cavity, and genital tract and can be isolated in cases of appendicitis, cholecystitis, peritonitis, pericarditis, osteomyelitis, and sepsis

Structure and Properties

Morphology

Bacteroides and related bacteria are gram-negative rods, albeit coccobacteria may occur. Certain microbial species can possess capsule and/or flagella. Fusobacteria render characteristic rod-like shapes with tapered ends.

Cultivation

All of these agents are anaerobic bacteria, but they develop different levels of oxygen tolerance. Some strains of bacteroids can survive at 1-2%

concentration of oxygen. Low oxidation-reduction potential promotes bacteroidal growth.

Usually they are cultivated on ***blood agar*** with yeast extract and other growth factors (e.g., *Schaedler anaerobe agar*) within anaerobic jars better in atmosphere of 5-10% CO_2. They grow slowly within 4-5 days. Some species (e.g. *P. melaninogenica*) produce black pigment.

The representatives of genus *Bacteroides* are resistant to bile salts and penicillin that distinguishes them from other similar bacteria.

Biochemical properties

Strict anaerobes have no cytochrome systems and possess neither catalase, no superoxide dismutase enzymes. Nevertheless, bacteroides members express small amounts of catalase and superoxide dismutase that partially preserve bacteria from reactive oxygen radicals.

Anaerobic bacteria gain energy from fermentation of different substrates. Bacteroids utilize vast number of carbohydrates producing the broad spectrum of acid end products. Also they metabolize pepton.

Antigenic structure

Antigenic structure is variable, depending on cell wall composition, capsule and flagella presence.

Virulence factors

Bacteroides produce ***destructive enzymes*** (hyaluronidase, collagenase, plasmin, heparinase, etc.) that cause tissue damage.

B. fragilis produces exotoxin ***fragilysin***. This toxin exhibits potent proteolytic activity destroying intercellular junctions between enterocytes.

Cell wall lipopolysaccharides can activate leukocyte chemotaxis, but they are almost lack of endotoxin activity. ***Capsule*** layer of bacteroides preserves them from phagocytosis.

Enzymes destroying antibacterial drugs (e.g., ***beta-lactamases***) confer microbial resistance to antibiotics. Also bacteroides are resistant to aminoglycosides and demonstrate growing resistance to tetracyclines.

Resistance

Non-sporeforming anaerobic bacteria demonstrate generally low resistance to external influences. They are killed by heating at 65^{o}C in 15 min, and in 1 min by boiling; when exposed to the opened air, they are irreversibly inactivated in 24-48 h. However, in feces they may stay viable up to 1 month.

Pathogenesis of Infections caused by Non-sporeforming Anaerobic Bacteria

Bacteroides species are the major part of normal microflora of large intestine. Bowel injury and/or the increase of permeability of intestinal wall induce microbial spread towards peritoneal cavity that results in abdominal purulent inflammation followed by peritonitis or intestinal abscessing.

B. fragilis demonstrates markedly enhanced virulence in comparison with other bacteroidal species. This pathogen represents only 0.5% of all microorganisms of large intestine, but it can be isolated in 30-60% of cases of anaerobic infections, especially in their intra-abdominal and wound localizations.

This bacterium possesses the number of potent virulence factors. Among them are ***agressive enzymes*** hyaluronidase, proteases, and hemolysin. Together with enterotoxin ***fragilysin*** that destroys intercellular contacts of enterocytes they promote microbial leakage across the intestinal wall thus stimulating microbial invasiveness and spread from their primary sites.

Capsular polysaccharide of *B. fragilis* triggers local pathological inflammatory response that leads to formation of tissue abscesses.

As the result, *B. fragilis* takes an active part in peritonitis, intestinal and liver abscesses, appendicitis, abscesses of subcutaneous adipose tissue, endometritis, vulvar abscesses, trophic ulcers in diabetes patients, lung abscesses, anaerobic infections of central nervous system (brain abscesses and subdural empyema),

Similar pathology can be caused by other bacteroidal species (*B ovatus*, *B vulgatus*, *B. thetaiotaomicron*, etc.)

Severe anaerobic infections are also caused by the members of *Bilophila* and *Fusobacterium* genera. For instance, *Bilophila wadsworthia* is isolated in 50% of cases of appendicitis.

Fusobacterium necroforum is relatively common in wound anaerobic infections. The rate of its isolation increases in systemic bacterial infections such as endocarditis, bacteremia and sepsis.

Overall, usually five and more of bacterial species can be isolated from inflammatory site, including facultatively anaerobic and anaerobic bacteria.

Oral cavity harbors large amounts of porphyromonads (*P. gingivalis*, *P. endodontalis* and others), prevotellas (*P. intermedia*, *P. melaninogenica*) and tannerellas (*T. forsythia*). In various combinations

with other microbial pathogens they actively contribute to progression of periodontites

Porphyromonads can be isolated also in suppurative bacterial infections of various localizations.

Likewise, members of prevotella species can affect female genital tract, causing pelvic inflammatory disease and tubo-ovarian abscesses.

Laboratory Diagnosis of Anaerobic Infections

The ***material*** is obtained in anaerobic conditions, e.g. by abscess puncture with syringe that contains appropriate medium for anaerobes (e.g., *thioglycolic* medium). The specimen should be transferred immediately into the sealed bottle with transport anaerobic medium and delivered to the laboratory within 1-1.5 h.

Specimens, obtained from closed purulent foci, blood and cerebrospinal fluid are examined.

Since various anaerobic species produce different spectra of short-chain fatty acids they can be identified by ***gas-liquid chromatography***. This method may be used for rapid diagnosis of anaerobic infection.

Also ***rapid identification*** of non-sporeforming anaerobic bacteria in clinical specimens is performed by serological (***ELISA***) and molecular genetic tests (***PCR***).

Anaerobes are ***cultivated*** upon blood agar, trypticase soy agar, brain-heart infusion agar and other enriched media in anaerobic jars. The jars are usually supplied with disposable packets that produce hydrogen, and a catalyst that combines the hydrogen with any free oxygen to form water. Cultures are incubated at 35-37°C with addition of CO_2.

The microbial isolates are further identified by their morphology, cultural properties and biochemical activities.

Treatment and Prophylaxis of Anaerobic Infections

Prophylaxis of anaerobic infections is *non-specific* and includes adequate surgical treatment followed by drainage that ensures sufficient oxygen access to affected site.

The most active antibiotics for ***treatment*** of anaerobic infections are *metronidazole* and *clindamycin*. Most of non-sporeforming anaerobic

bacterial strains are shown to develop no resistance to clindamycin and metronidazole. In severe cases of anaerobic infections carbapenems are successfully used. Sensitive strains are treated with other β-lactam antibiotics (e.g., penicillins and cephalosporins).

Chapter 3
CAUSATIVE AGENTS OF ENTERIC BACTERIAL INFECTIONS: *ESCHERICHIA COLI* AND SHIGELLAE

PATHOGENIC ESCHERICHIAE

General Characteristics and the History of Discovery

The typical member of *Enterobacteriaceae* family *Escherichia coli* is a widespread normal inhabitant of human intestinal tract. Escherichiae are permanently discharged into the environment from the gut of mammals, birds, amphibians and many other organisms. These bacteria can be isolated from water, soil and different foodstuffs, including dairy products. *Escherichia coli* and other coliform bacteria are defined as sanitary indicator microorganisms for these objects.

German scientist T. Escherich discovered *E. coli* in 1885.

Classification

The family Enterobacteriaceae comprises about 50 genera. Among them *Escherichia, Shigella, Salmonella*, *Yersinia*, *Klebsiella*, *Proteus, Morganella, Providencia* are regarded as the most important in clinical practice.

Escherichia genus encompasses at least 6 closely related species. Besides *E. coli*, *Escherichia vulneris* can be uncommonly found in wound infections and *E. hermannii* is rarely isolated from wounds, blood or cerebrospinal fluid (CSF).

Escherichia coli species consists of several biotypes and great number of serotypes, which are discerned by their biochemical and antigenic properties.

Structure and Properties of *Escherichia coli*

Morphology

Escherichia coli are small or middle-size gram-negative rod-shaped bacteria 1-3 μm in length. During microscopy they are usually observed as single cells.

E. coli carry peritrichous flagella; nevertheless, non-motile microbial forms can be found. They possess numerous pili that promote microbial adherence, nutrition and gene exchange. Escherichiae as well as other enterobacteria have no spores, but can produce capsule.

Cultivation

All escherichiae can grow easily on basic nutrient media within standard temperature range 10-45°C at pH of 7.2-7.6.

The growth on meat peptone agar (MPA) renders round convex smooth semi-transparent colonies. Meat peptone broth (MPB) cultivation results in diffuse turbidity followed by cell precipitation.

As *E. coli* in most of cases utilize lactose, they form ***lactose-positive colonies***, which are red on ***McConkey*** agar, ***Endo*** agar, and dark-blue on ***eosin-methylene blue*** (***EMB***) agar (i.e., ***Levine*** medium).

Some *E. coli* strains produce hemolysin.

Biochemical properties

E. coli are facultative anaerobes with mixed type of metabolism. They ferment great number of carbohydrates (glucose, lactose, maltose, mannitol, galactose, and very rarely sucrose) with acid and gas end products. They metabolize proteins with indole formation, express lysine decarboxylase and reduce nitrates to nitrites.

As all other members of the family *Enterobacteriaceae*, *E. coli* are oxidase-negative, but catalase-positive microorganisms.

Antigenic structure

E. coli carries a large variety of antigenic determinants of different origin.

Somatic lipopolysaccharide cell wall antigen, or ***O-antigen***, is heat-resistant and withstand heating up to 80-100^{o}C. It demonstrates ***endotoxin*** activity due to ***lipid A*** moiety, while antigenic epitopes contain predominantly various carbohydrate residues and aminosugars.

O-antigen is regarded as group specific.

Flagellar ***H-antigen*** of *E. coli* contains protein ***flagellin***, which is heat-sensitive and can be destroyed at a temperature above 56^{o}C.

Capsular, or ***K-antigen*** is composed of complex polysaccharides. It covers cell wall O-antigen and preserves it against the actions of phagocytes and antibodies.

K-antigen of capsular *E. coli* strains displays evident structural variations: heat-stable A-fraction and heat-labile L- and B-fractions.

The complete antigenic structure of *E. coli* is very individual. Different O-, H-and K-antigens appear in various combinations in particular bacterial strains.

More than 170 serogroups based on O-antigen, about 100 types of K-antigen and more than 50 types of H-antigen are known to date. Complete antigenic formula includes the certain O-, H-and K-antigens of the questioned strain (e.g., O26: K60(B6): H2). Antigenic structure of *E. coli* can be modified by genetic recombinations and mutations that affect the bacteria.

Virulence factors

Most of escherichia strains are ***non-pathogenic***, being the representatives of normal human intestinal flora. Some pathogenic isolates (***virotypes***) possess virulence factors, encoded predominantly by plasmids or temperate bacteriophages. The bacteria produce the number of *enterotoxins*, *hemolysins* and ***cytotoxins*** (e.g., ***verotoxins*** or ***Shiga-like toxins SLT I*** and ***SLT II***). Certain virotypes express the structures of type III secretion system (T3SS) or ***injectisome*** responsible for microbial invasiveness, spread and intracellular persistence. All microbial cells have the vast number of adhesion molecules.

Cell wall LPS exhibits ***endotoxin*** activity. Many of *E. coli* strains produce bacteriocins (***colicins***), thereby affecting neighboring microflora.

Resistance

E. coli stays viable for several months in different environmental conditions. These bacteria are inactivated at temperature of 60°C within 15 minutes and rapidly destroyed by boiling. They are susceptible to most of hospital disinfectants and antiseptics taken in standard concentrations.

Pathogenesis and Clinical Findings of Infections Caused by *E. coli*

The diseases, caused by various escherichia isolates, are generally termed "***escherichioses***".

Non-pathogenic and facultatively pathogenic *E. coli* ensure normal intestine functions, taking part in nutrition, cellulose digestion, vitamin synthesis, peristaltic regulation, etc. Nevertheless, they can provoke pathology after colonization of unusual biotopes, such as urogenital or biliary tract, peritoneal cavity or central nervous system. In case of

patient's immunodeficiency the generalization of infectious process is possible, resulting in septicemia.

Non-specific or ***opportunistic*** *E. coli* infections affect predominantly urinary tract. *E. coli* is the main causative ***agent of urinary infections***, especially in young women. These bacteria are shown to produce ***hemolysins***.

E. coli, expressing pili of certain type (***P pili***), become associated with ***pyelonephritis***, since P pili promote microbial adhesion to epithelium of urinary tract. Bacterial adherence to uroepithelium is followed by microbial degradation with the release of LPS endotoxin. It leads to neutrophil infiltration, cytokine overproduction and progression of inflammatory response.

E. coli strains are often isolated in patients with cholecystitis, cholangitis, appendicitis, peritonitis and other abdominal diseases.

Also *E. coli* is an important cause of ***infant meningitis***. It mainly affects premature newborns and infants under the age of 1 month. Most of causative agents pertain to the specific serovar O18:K1 as K1 capsular antigen is resistant to complement action. In addition, these bacteria have special *S-fimbriae* with elevated tropism to endothelial cells of CNS blood vessels. The disease is extremely severe; the mortality rate may exceed 50%.

Specific E. coli infections comprise a large number of ***diarrheal diseases***. They are caused by pathogenic escherichia strains.

These disorders have the specific mechanisms of the development resulting from the action of various *E. coli* toxins.

Infection are transmitted by ***fecal-oral route*** (foodborne, waterborne or through contaminated fomites).

The main ***sources of infection*** are *sick persons* or *carriers*. In situations with enterohemorrhagic diarrheas there can be carrier *animals* (e.g., cattle).

Enteropathogenic E. coli (***EPEC***) affect infants worldwide.

EPEC ***coli-enteritis*** is caused by numerous serotypes of *E. coli*. EPEC reveal a distinct capacity of adherence to intestinal cells.

Intestinal colonization by EPEC is promoted by interaction of microbial adhesin ***intimin*** with its specific receptor ***Tir***. This process is mediated by bacterial ***type III secretion system*** (*injectisome*) that initially translocates an intimin receptor Tir into the host cell (see below).

As the result, membrane cup-like filamentous pedestals enwrapping each bacterium are formed that is followed by destruction of local microvilli. This is known as an *attaching and effacing* (A/E) *phenomenon*.

All of the genes essential for induction of A/E lesions in EPEC are confined within specific "***pathogenicity island***" termed as the ***locus for enterocyte effacement***.

Devastation of epithelial villi results in profound watery diarrhea.

Enterotoxigenic E. coli (***ETEC***) is the common causative agent of diarrhea in developing countries. Also it causes so-called "*traveller's diarrhea*". The ***infectious dose*** here is relatively high – about 10^6-10^9 microbial cells.

ETEC infection results from the action of ***heat labile exotoxin*** (***enterotoxin***) with molecular weight of 80,000 that is very similar with cholerogen of *Vibrio cholerae*.

B-subunit of enterotoxin is absorbed to the intestinal cells via cell membrane ganglioside receptor. Subunit A enters into the cell across the membrane promoting ADP-ribosylation of cellular G-proteins. This event activates guanylate cyclase and adenylate cyclase resulting in abnormal ***increase of cAMP*** concentration inside the cells. The latter event stimulates secretion of chlorides into the small intestine with impairment of sodium and water absorption. As the result, massive ***non-inflammatory diarrhea*** evolves.

Certain isolates express several ***heat stable enterotoxins***. Co-expression of both toxin types results in more profound diarrhea.

Enterotoxins, as well as colonization factors of ETEC, are encoded predominantly by plasmid genes.

Heat labile exotoxin elicits the synthesis of antitoxic antibodies, which possess neutralizing activity.

Enteroinvasive E. coli (***EIEC***) reveal the striking ability to invade intestinal epitheliocytes with ***intracellular propagation*** that is followed by microbial lateral spread towards adjacent neighboring cells. The enteroinvasive disorders are very similar to shigellosis caused by *Shigella flexneri*, *S. boydii* and *S. sonnei*. EIEC are almost identical to shigella (non-motile bacteria, which are lack of lactose fermentation) but deprived of ability to produce Shiga STX toxins.

Enteroaggregative E. coli (***EAEC***) promote diarrhea due to their strong adhesive capacity to intestinal cells with tendency of self-aggregation. The mode of their adhesion is not similar with adherence pattern of EPEC.

EAEC binding was primarily determined as diffuse adherence, but further two main patterns were observed: ***aggregative adherence*** and ***diffuse adherence***. Bacteria express two types of specific fimbriae: *aggregative adherence fimbriae I* and *II* (*AAF/I* and *AAF/II*, respectively).

Usually EAEC don't produce toxins. Nevertheless, they damage intestinal cells and hamper the normal exchange of water and electrolytes within the bowel, thus causing a chronic or persistent form of diarrhea with duration of more than 14 days.

Enterohemorrhagic E. coli (***EHEC***) are the most dangerous representatives of coliform bacteria. They cause severe ***hemorrhagic colitis*** with diarrhea and intestinal cell destruction. The infection outcome becomes much more serious with the development of life-threatening ***hemolytic uremic syndrome*** (***HUS***) that is often followed by acute renal failure.

The ***incubation period*** of the disease endures about 5-7 days.

The ***infectious dose*** of bacteria is ***extremely low*** (1-100 cells).

For a long time *E. coli* of ***O157:H7*** serotype was considered to be the major pathogenic variant of EHEC. Now it is obvious that STEC strains causing human disease, pertain to a very broad range of O:H serotypes (more than 30 O-serogroups with multiple antigenic variants are known to date, and this list is being increased permanently).

High virulence of EHEC depends on production of adherence factors and potent cytotoxins.

The ability of EHEC as well as EPEC to attach to and efface enterocytes results mainly from the activity of *E. coli* outer membrane protein "***intimin***" and its ***translocated receptor Tir***. Both proteins are encoded by genes located in the same "*locus of enterocyte effacement*" within ***pathogenicity island*** of bacterial chromosome.

Before tight bacterial attachment, Tir receptor protein is injected into intestinal cells via type III bacterial secretion system (***needle complex***, or ***injectisome***). Once expressed on enterocyte membranes, Tir interacts with microbial intimin that ensures the strong binding of *E. coli* to intestinal cells.

EHEC secrete two main types of ***Shiga-like toxins*** (***SLT I*** and ***SLT II*** or ***verotoxins***), which are very similar with *Shigella dysentheria* exotoxin Stx (SLT I toxin is almost identical). Toxin production in EHEC is encoded by temperate bacteriophages.

SLT toxin of EHEC is composed of A and B subunits. Protein A-subunit of 32 kDa is noncovalently bound to five 7-kDa B-subunits. B-subunit promotes attachment to eukaryotic cell receptor ***glycolipid Gb3*** (or *globotriosyl ceramide*). The cells, bearing Gb3 receptor, are susceptible to SLT toxin action. When Shiga-like toxins appear in the bloodstream, they induce the damage of glomerular endothelial cells of kidneys, which express large amounts of the Gb3 receptor.

After receptor binding, toxin molecules are internalized by receptor-mediated endocytosis. Subunit A is further cleaved into two fragments, where A1 portion of toxin renders ***RNA N-glycosidase activity***. A1 fragment breaks down *N*-glycosidic bond in the 28S rRNA, thus preventing aminoacyl-tRNA binding to the 60S subunit of ribosome. These molecular events ***terminate elongation of protein sequence*** and eventually cause cell death.

Additional virulence factors encompass several ***enterohemolysins*** and ***extracellular serine protease***, which cleaves human coagulation factor V, thereby maintaining hemorrhagic colitis.

Multiple devastating activities of EHEC virulence factors promote destructive ***hemocolitis*** with severe diarrhea.

The disease tends to be self-limited, though ***hemolytic uremic syndrome***, followed by hemolytic anemia and thrombocytopenia, can develop in 10-30% of cases and even more. It is one of the leading causes of renal failure in children. The mortality rate in HUS is near 5%.

Immunity reactions after escherichia infections are usually group specific and low grade. Natural passive immunity conferred by maternal milk sIgA can protect newborns and infants against coli-enteritis for several months after birth. Similarly, trans-placental IgG-mediated immunity defends infants against infections, caused by some enteroinvasive escherichia strains.

Normal microflora of gastrointestinal tract (e.g. bifidobacteria and lactobacilli) promotes powerful non-specific host defense due to their substantial antagonistic activity against pathogenic enterobacteria.

Laboratory Diagnosis of Coli-Enteritis and Other *E. coli* Infections

Specimens for diagnosis of ***non-specific escherichioses*** are obtained from the site of infection: urine, bile, blood, pus, or wound discharge are examined.

For laboratory diagnosis of ***escherichia-associated diarrhea*** feces, vomiting masses, food remnants, water, washing samples, etc. should be examined.

For ***rapid identification*** of pathogenic E. coli in clinical specimens molecular genetic tests are applied (e.g., ***PCR*** test).

Microscopical tests are useless owing to the evident morphological similarities of all enterobacteria.

To confirm the diagnosis of coli-enteritis, ***isolation of microbial culture*** is elaborated.

To aim this, the specimens are plated upon the differential or selective nutrient media (McConkey agar, EMB or Endo medium). The growth of colored ***lactose-positive*** colonies is evaluated.

To determine the nature of grown *E. coli* isolates, the tentative slide agglutination test with polyspecific OK-serum against the most widespread enteropathogenic *E. coli* is performed. At least 10 lactose-positive colonies should be investigated. In case of positive results the rest of the colony is planted on slant MPA to obtain pure culture. It is identified by slide agglutination tests with different serovar-specific OK-sera.

Positive result of slide agglutination with type-specific OK-serum is confirmed by extended tube agglutination test. To establish the concordance of isolated culture to serum specifity and titer, the reaction is performed separately for O- and K-antigens (with boiled and native culture, respectively).

Evaluation of biochemical properties, phagotyping and antibiotic susceptibility tests accomplish culture examination.

To reveal enterohemorrhagic *E. coli*, the specimens are planted on modified McConkey agar that contains ***sorbitol*** instead of lactose. EHEC 0157:H7 are ***sorbitol-negative***, whereas other escherichia are usually sorbitol-positive on MacConkey agar.

Shiga-like toxins as well as escherichia enterotoxins are determined by ELISA, cell culture tests or molecular genetic methods in reference laboratories.

Treatment and Prophylaxis of *E. coli* Infections

Various groups of antibiotics (amoxycillin, third-generation cephalosporins, aminoglycosides, or fluoroquinolones) are used for ***treatment*** of opportunistic infections, caused by escherichiae.

In patients with *E. coli*-associated diarrheas an adequate antibiotic therapy shortens the diarrheal period, but the microbial resistance rapidly increases under antibiotic pressure.

Antibacterial treatment should be administered with great precautions in patients with hemorrhagic colitis and HUS. The drugs affecting metabolism of microbial nucleic acids (e.g., fluoroquinolones and co-trimoxazole) are not recommended here as they may stimulate the spread

of virulence genes among the enterobacteria. In these clinical situations carbapenems are regarded as the most suitable antimicrobial agents.

The disease therapy with probiotics (e.g., lactobacterin, bificol, bifidumbacterin) is also beneficial.

Specific prophylaxis *is not available* for *E. coli* infections. The tight control of sanitary conditions, prevention of water and foodstuff contamination, maintaining of hygiene standards is of great importance.

SHIGELLAE

The History of Discovery

The agents of bacterial dysentery were first discovered by Chantemesse in 1888. In 1898 they were thoroughly studied by K. Shiga. A novel causative agent of dysentery was described later by S. Flexner and co-workers in 1900.

At the borderline of 1900s some other agents of bacterial dysentery were isolated. K. Duval in 1904, as well as V. Kruse and co-workers in 1907, and K. Sonne in 1915 revealed the species, able to ferment lactose unlike the previously described isolates.

Finally all these microbial representatives were placed into separate genus called *Shigella* in honor of K. Shiga.

Shigellae cause *bacterial dysentery* or ***shigellosis***. This infection circulates predominantly among the population of developing countries, affecting near to 150 million people annually with more than 1 million death cases. About 70% of patients are 1-5-year-old children.

Classification

Shigella genus pertains to the family *Enterobacteriaceae*. The genus contains 4 species: *S. dysenteriae* of group A, *S. flexneri* (group B), *S. boydii* (group C), and *S. sonnei* (group D).

Different shigella species comprise numerous biotypes and serovars.

Genetic analysis revealed that all shigellae share more than 90% of genomic DNA sequence with escherichiae. Therefore, they can be accounted as single genomospecies. However, the evident phenotypic

dissimilarities of these bacteria, largely dependent on acquisition of various mobile genetic elements, result in striking differences in their virulence for humans. Hence, they remain to exist as separate genera and species.

Structure and Properties of Shigellae

Morphology

All shigella closely resemble other *Enterobacteriaceae* members: small gram-negative non-motile rods without spores, possessing multiple pili. Certain strains can form thin capsule.

Cultivation

These bacteria readily grow on basic nutrient media with optimal temperature about 37°C, pH 7.2.

The growth reveals round small convex transparent colonies. In meat peptone broth shigellae produce homogenous turbidity.

After cultivation in lactose-containing media (***McConkey agar***, or Ploskirev's medium) most of shigellae form ***lactose-negative*** transparent colonies. *S. sonnei* can slowly ferment lactose.

Biochemical properties

Shigellae are facultatively anaerobic bacteria. They utilize various carbohydrates with acid formation. Individual biovars (*Newcastle* subsp.) can produce small amounts of gas. All shigellae ferment glucose. Most of bacteria, except *S. dysenteriae,* ferment mannitol. *S. sonnei* can metabolize lactose and sucrose within several days.

The bacteria can't produce hydrogen sulfide, but certain strains display proteolytic activity with indole formation.

Antigenic structure

Shigellae are classified into 4 groups according to their antigenic properties. These groups comprise more than 40 serotypes.

All of these bacteria contain group-specific O-antigen, some isolates produce capsular K antigen.

Somatic lipopolysaccharide O-antigen possesses endotoxin activity.

Virulence factors

Pathogenic shigellae produce large number of virulence factors, responsible for microbial adherence, invasiveness, intercellular spread, apoptosis of host immune cells, and intestinal epithelium death that results in severe inflammation of large intestine with ***hemocolitis***.

Bacterial invasion is controlled by special structures of ***type III secretion system*** (so-called ***secreton III***), which includes bacterial ***injectisome***, or ***needle complex***. Once attached to the host cells via ***needle complex***, bacteria inject the number of invasion proteins into target cell. These ***effector proteins*** re-build cytoskeleton of affected cell, thus promoting intercellular microbial spread and further invasion.

All virulent *Shigellae* contain a ***large*** 220-kb ***plasmid*** harboring pathogenicity island that determines the "invasive phenotype" of bacteria. Invasive proteins are encoded predominantly by ***ipa/spa*** (***invasive plasmid antigen***) genetic locus.

Deep damage of bowel epithelium is promoted by cytotoxic action of bacterial ***Shiga toxin*** (***STX toxin***), which is encoded by chromosomal ***stx*** gene. Maximal toxin production is essential for *S. dysenteriae* type 1.Toxin action pattern is very similar with enterohemorrhagic *E. coli* verotoxins.

As in EHEC, STX toxin is composed of A and B subunits. Several receptor B-subunits bind to cellular receptor glycolipid Gb3. Exotoxin internalization is followed by subunit A cleavage. Toxic A1 fragment possesses RNA *N*-glycosidase activity and thereby cleaves *N*-glycosidic bond within 28S ribosomal RNA. ***Termination of protein synthesis*** causes the death of host cells.

LPS-containing ***endotoxin*** of shigellae activates phagocytes and other immune cells that is followed by exuberant cytokine release and tissue inflammation.

Resistance

Shigellae are not the highly resistant bacteria, but they can survive in the environment within 5-10 days. The most resistant is *S. sonnei* that keeps viability for months in water and different foodstuffs, e.g. dairy products.

Bacteria are killed by heating at 56°C within 10-15 minutes and inactivated readily by standard medical disinfectants (chloramine, hypochlorite, phenol, etc.)

Pathogenesis and Clinical Findings in Shigellosis

Different clinical forms of *bacterial dysentery* or ***shigellosis*** are caused by enteroinvasive shigellae.

The disease is transmitted by ***fecal-oral route*** and direct contact. It is "food, fingers, feces, and flies"-transmitted disorder.

Water outbreaks of shigellosis are related with *S. flexneri*, while foodborne disease cases ensue from *S. sonnei* infection. The disease caused by *S. dysenteriae* is particularly severe.

The main ***sources of infection*** are the *patients* with dysentery and bacterial *carriers*. The disease affects only humans.

Incubation period lasts from 1 to several days.

Infectious dose of 10-100 microbial cells is enough to cause the disease in adults (e.g., for *S. dysenteriae* infection).

Some shigellae are killed, passing through the stomach. The rest of bacteria comes to the bowel and invade the colon mucosa. Bacteria are specific to the rectal and large intestine mucous membranes.

The main intestinal entry site for shigellae is the ***follicle-associated epithelium*** that covers the mucosa-associated lymph nodes. Special epithelial ***M cells*** (*microfold cells*) are the primary targets for microbial invasion. After cell contact with bacterial needle complex, ***IpaB*** and ***IpaC*** proteins create a pore in eukaryotic cell membrane, and invasive proteins are injected inside the target cells. They trigger intracellular actin polymerization that results in membrane pocket formation. This way M-cells engulf and translocate shigellae into the cytoplasm. Bacterial ***VirG protein*** activates cell actin attachment to the pole of microbial cell with formation of ***actin comet***. Comet bacterial cell is able to move within the infected cells and can readily achieve the neighboring enterocytes ("*lateral spread*" of shigellae) thus promoting further microbial invasion.

Intestinal macrophages become invaded in similar manner. Invasive IpaB protein induces macrophage release of most potent ***proinflammatory cytokines*** IL-1 and IL-18 and at the same time triggers phagocyte death via *caspase 1-mediated* ***apoptosis***, thereby preventing shigellae from the death within macrophages.

Inflammatory cytokines cause the injury of intestinal wall. But at the same time they activate immune inflammation, attracting neutrophils to the invaded bacteria. Efficient leukocyte reaction restricts the infection up to its termination.

Massive cytolysis of intestinal epithelium is promoted also by ***Shiga cytotoxin*** action.

All these events lead to severe colon destruction resulting in ***hemorrhagic colitis***. General intoxication is followed by abdominal pain, fever, and hemorrhagic diarrhea with water loss. Intermittent painful rectal spasm (or ***tenesmus***) is characteristic for developed shigellosis.

The disease can be self-restricted within several days, but profound dehydration and acidosis require urgent therapy and even can cause lethal outcome especially in children.

The immunity acquired after the dysentery is group- and type-specific but relatively weak and of a short duration. For this reason the disease may recur many times and in some cases may become chronic.

Shiga toxin as a potent antigen elicits the synthesis of neutralizing antibodies.

Laboratory Diagnosis of Shigellosis

Reliable results of laboratory examination depend on correct ***sampling of stool specimen*** and its immediate inoculation onto a selective and differential medium at the patient's bedside. The inoculated material should be rapidly delivered to the laboratory.

As an example, the clinical specimen (feces) should be best collected directly from patient’s rectum by rectal swab and planted immediately after the collection onto ***McConkey agar***, ***EMB*** or ***Ploskirev’s*** medium.

Ploskirev’s medium contains meat-peptone agar, lactose, indicator neutral red, and bile salts with brilliant green dye to suppress concomitant microflora. The similar composition is of McConkey agar.

The plates are incubated at 37°C for 24 hours. When growing, shigellae produce ***lactose-negative*** transparent colonies.

The culture is further ***isolated*** in butt-slant double sugar agar (***Russel’s*** medium). It contains meat-peptone agar, 1% lactose, 0.1% glucose, and indicator dye. Inoculation of bacteria is performed both in aerobic and by stab in anaerobic conditions. As the result, the color change will appear only in the butt of medium due to glucose fermentation in anaerobic conditions. The slant part of agar would be intact because the most of shigellae are lactose-negative.

The pure culture obtained is further identified according to its biochemical and serological properties. In latter case the culture is tested by agglutination reaction with specific sera.

For ***rapid identification*** of ***DNA*** of shigellae species in clinical specimens sensitive and reliable molecular ***genetic tests*** are applied (e.g., ***PCR***).

Serological examination has no value in diagnosis of shigellosis.

Treatment and Prophylaxis of Shigellosis

Taking into account the increased resistance of shigellae to the long list of antimicrobial agents (e.g., ampicillin, trimethoprim-sulfamethoxazole, doxycycline, or chloramphenicol) *fluoroquinolones* (norfloxacin) and *third generation cephalosporins* (e.g., cefotaxime or ceftazidime) are most commonly used now for ***treatment*** of shigellosis.

Important measures of supportive symptomatic treatment include *urgent infusion therapy* to compensate water and electrolyte loss. The treatment of shigellosis with *probiotics* restores the normal composition of intestinal microflora.

Efficient vaccines for prevention of shigellosis are not available yet.

Non-specific prophylaxis of the disease comprises thorough control of water and food microbial contamination, isolation and adequate treatment of patients with laboratory confirmation of the recovery, the detection and treatment of carriers, adequate disinfection measures, the maintenance of sanitary and hygienic regimens according to the actual regulation acts, etc.

Chapter 4

CAUSATIVE AGENTS OF ENTERIC BACTERIAL INFECTIONS: SALMONELLAE AND YERSINIAE

SALMONELLAE

The History of Discovery

In 1880 German scientist K. Eberth first described the bacterium – causative agent of enteric typhoid fever. Later in 1884 it was isolated and thoroughly investigated by G. Gaffky.

Causative agent of similar disease, paratyphoid fever, was initially isolated by C. Archard and R. Bensaude and studied in details by H. Schottmuller in 1900. It was named later *Salmonella paratyphi B* or *S. schottmuelleri*. Another paratyphoid bacterium, or *S. paratyphi A* was investigated by A. Brion and H. Kayser in Germany.

All these salmonellae were found to cause the diseases in humans. Also many salmonella species were isolated in animal diseases. D. Salmon in 1885 revealed the causative agent of pig's plague, *S. choleraesuis*. Then various salmonellae of animal origin were demonstrated to cause food poisoning, or toxinfections in humans. In particular, in 1888 A. Gartner isolated *S. enteritidis* both from cow's meat and patient, died from toxinfection. In 1896-1898 K. Kensche and E. Nobel discovered another significant agent of food poisoning, *S. typhimurium*. Finally, it was proven that great number of salmonella species, isolated from animals, can cause human food poisoning and in some cases septicemia.

Classification

Salmonellae pertain to the family *Enterobacteriaceae*. In past more than two thousand species were described within *Salmonella* genus. Recent genetic studies revealed only two species of salmonellae – *S. enterica* and *S. bongori* with vast number of antigenic salmonella variations.

S. enterica species is further divided into several distinct subspecies.

S. enterica subsp. enterica comprises more than 99% of salmonella that cause diseases in humans.

The complete name of distinct salmonella isolates includes species name and the name of serovar (former species designation). Antigenic variant (***serovar***) is designated with starting upper-case letter and non-italicized straight font. For instance, causative agent of enteric typhoid fever is classified as *S. enterica* serovar Typhi or S. Typhi for short.

Limited number of salmonella serovars affects humans only. Most serious disease is ***enteric typhoid fever***, caused by S. Typhi.

S. Paratyphi A, S. Paratyphi B and S. Paratyphi C are the agents of ***paratyphoid enteric fevers***. The latter diseases are generally regarded as anthroponoses, but these bacteria may be also isolated from animals.

Numerous salmonella serovars are the causative agents of ***salmonelloses***. Usually salmonelloses are contracted from animal sources and appear in two major clinical forms – ***food poisoning*** (***food toxinfection***) and ***septicemia***. Septicemia as more rare but severe clinical condition predominantly affects children. S. Typhimurium and S. Enteritidis are the most virulent and frequently isolated agents, causing these infections. Many other variants (e.g., S. Choleraesuis, S. Derby, or S. Heidelberg) can also cause salmonelloses.

Structure and Properties of Salmonellae

Morphology

All salmonellae are very similar. Their morphology is typical for *Enterobacteriaceae* family members (gram-negative small or medium-size rods without spores).

Salmonellae possess peritrichous flagella and multiple pili. Virulent strains carry needle complex, or injectisome – type III secretion system structures.

The strains, isolated from carriers, frequently produce capsule-like polysaccharide substance.

Cultivation

Salmonellae easily grow on basic nutrient media within temperature range from 15 to 40°C with optimum of 37°C at pH 7.0.

The growth in meat peptone agar results in round semitransparent middle-size colonies. S. Paratyphi B colonies produce edge mucous swelling.

Salmonellae are resistant to bile salts and number of antiseptics, e.g. brilliant green, sodium selenite or sodium tetrathionate. Thus, they are

cultivated on various selective and enrichment media that inhibit *E. coli* growth. Among them are meat-peptone ***broth with bile salts***, ***selenite*** broth, ***tetrathionate*** broth, ***Wilson-Blair agar*** (i.e., ***bismuth sulfite agar***), composed of MPA, glucose, bismuth sulfite, ferrous sulfate and brilliant green.

Growing on *bismuth sulfite agar*, salmonella produce black colonies due to the formation of iron sulfide, except serovar S. Paratyphi A.

As salmonellae are lack of lactose fermentation, they form ***lactose-negative colonies*** on McConkey agar, EMB (Levine) agar, etc.

Biochemical properties

Salmonella are facultative anaerobes.

S. Typhi ferments various carbohydrates (glucose, maltose, mannitol, dextrin, glycerol and others) with ***acid production***. Other salmonellae, e.g. S. Paratyphi A and B, S. Typhimurium, S. Enteritidis, etc., utilize carbohydrates with ***acid*** and ***gas*** end products. All salmonella are ***lactose-negative*** bacteria.

Pathogenic salmonellae, except *S. paratyphi A*, reveal proteolytic activity with ***hydrogen sulfide*** formation. They reduce nitrates to nitrites.

As all the members of *Enterobacteriaceae* family, salmonellae are oxidase-negative, but catalase-positive bacteria.

Antigenic structure

Salmonellae possess somatic O- and flagellar H-antigens. S. Typhi strains, predominantly isolated from microbial carriers, synthesize outer capsule-like Vi-antigen. Temperate phage transduction can influence the expression of salmonella antigens.

Lipopolysaccharide heat-stable ***O-antigen*** displays endotoxin activity. Flagellar ***H-antigen*** is heat-labile. Polysaccharide complex ***Vi-antigen*** is also a heat-labile substance. It is readily destroyed by boiling for 10 minutes.

Vi-antigen partially covers O-antigen, and thereby hampers microbial agglutination by anti-O antibodies. It is almost solely found in S. Typhi strains and in rare cases in S. Paratyphi C and S. Dublin.

Polysaccharides of Vi-antigen bind to the vast number of specific bacteriophages. As the result, about 100 of distinct phagotypes are determined in Vi-Ag-expressing S. Typhi.

F. Kauffmann and P. White elaborated the classification of salmonellae according to their O- and H-antigen variations (see Table 3). ***O-antigen*** is shown to be the ***group specific***. It is heterogeneous and

contains specific and several non-specific antigenic determinants. About 70 serogroups were distinguished by specific fraction of salmonellae O-antigen.

H-antigen is found to be in two phases, encoded by different genes.

Only ***first phase*** of H-antigen appears to be "species"- or ***variant-specific***. Phase 2 antigens are agglutinated by group-specific sera. More than 2500 serovars of salmonellae are identified by specific phase H-antigen.

Thus, serological typing of certain salmonella strains, in spite of their tremendous diversity, is reduced to simple two-step procedure: once the serogroup was determined by specific O-antigen agglutination, serovar identification is accomplished by agglutination with specific anti-H serum.

Table 3

Serological classification of salmonellae (by F. Kauffmann and P. White)

Serovar	**Serogroup**	**Antigens**		
		O	**H**	
			Phase 1	**Phase 2**
S. Paratyphi A	2 (A)	1, 2, 12	a	1, 5
S. Paratyphi B	4 (B)	1, 4, 5, 12	b	1, 2
S. Typhimurium		1, 4, 5, 12	i	1, 2
S. Derby		1, 4, 5, 12	f, g	1, 2
S. Heidelberg		1, 4, 5, 12	r	1, 2
S. Paratyphi C	7 (C1)	6, 7 (Vi)	c	1, 5
S. Choleraesuis		6, 7	c	1, 5
S. Isangii		6, 7, 14	d	1, 5
S. Infantis		6, 7, 14	r	1, 5
S. Newport	8 (C2-C3)	6, 8, 20	e, h	z, 2
S. Typhi	9 (D1)	9, 12 (Vi)	d	–
S. Enteritidis		1, 9, 12	g, m	–
S. Dublin		1, 9, 12 (Vi)	g, p	–
S. Moscow		9, 12	g, q	–
S. Panama		1, 9, 12	e, v	1, 5
S. Gallinarum		1, 9, 12	–	–
S. London	3, 10 (E1)	3, 10 [15]	l, v	1, 6
S. Anatum		3, 10 [15, 34]	c, h	1, 6

Virulence factors

Salmonellae produce various virulence factors that actively participate in disease pathogenesis. Since bacteria can persist intracellularly, they express multiple adhesins and invasive proteins, which promote microbial invasion, intercellular spread, and final impairment of host cellular immune response.

At least 10 genetic ***salmonella pathogenicity islands*** (***SPI***) are detected that encode microbial virulence factors. They are found both in bacterial nucleoid and plasmids. Many of them were delivered to bacterial cell with temperate bacteriophages upon transduction. Besides, S. Typhi harbors additional genetic element known as ***major pathogenicity island***.

Genes located in chromosomal pathogenicity islands SPI-1 and SPI-2 as well as in major pathogenicity island of S. Typhi play a crucial role in pathogenesis of salmonella-associated infections.

Genes of ***SPI-1*** and ***SPI-2*** code for the structures of ***type III secretion system*** with bacterial ***needle complex*** or ***injectisome***. By means of injectisome, salmonellae deliver invasive ***effector proteins*** into intestinal cells and phagocytes.

Genes of ***major pathogenicity island*** encode the capability of S. Typhi to produce ***capsular Vi-Ag*** that promotes microbial survival in worsen surroundings (e.g., within phagocytes or in gallbladder of carriers).

One of the most potent virulence factors of salmonellae is thermostable ***LPS endotoxin***. It activates macrophages and T cells that is followed by proinflammatory cytokine release and subsequent tissue damage. Endotoxin action provokes deep disorders of patient's gastrointestinal tract, cardiovascular system and CNS. Bacteria of typho-paratyphoidal group can produce large amounts of endotoxin.

Certain salmonella serovars, e.g *S. enteritidis*, are able to produce potent ***enterotoxin***. It activates enterocyte adenylate cyclase elevating intracellular cAMP concentration that results in diarrhea with massive secretion of water and chlorides into intestinal lumen.

Several genetic regions within nucleoid and plasmids of salmonellae contain genes of ***multidrug resistance to antibiotics***.

Resistance

Salmonellae reveal marked stability in the environment. They can survive for several weeks and even months in soil, contaminated by bacteria, as well as in various foodstuffs, where they can propagate (dairy products, meat, bread, etc.) The bacteria stay viable upon contaminated

fruits and vegetables up to 1-2 weeks. In water they maintain viability for 3-4 months. Salmonellae readily withstand drying and long-time freezing.

S. Typhi and S. Paratyphi A are inactivated at 56°C within an hour, while other bacteria are relatively resistant to heating at 60-70°C. Boiling rapidly inactivates bacteria. Nonetheless, microbial endotoxin is heat-stable and can cause food poisoning even in absence of live salmonellae.

S. Typhi is sensitive to conventional disinfectants (e.g., chlorine-containing chemicals or phenol.)

Pathogenesis and Clinical Findings in Typho-Paratyphoid Diseases

Enteric typhoid fever is the ***anthroponotic*** disease caused by S. Typhi and transmitted by ***fecal-oral route***. In developed countries it occurs as sporadic infection. Nevertheless, from 15 to 30 million disease cases appear annually worldwide. The disease spreads predominantly in developing countries. It results in 250,000-500,000 lethal outcomes being the serious public health problem.

Salmonella ***carriers*** and the patients with subclinical forms of illness are the main ***sources of infection***.

Infectious dose of S. Typhi is 10^3-10^5 microbial cells, i.e. it is rather low.

Incubation period lasts for about 10-14 days.

Salmonellae, entering gastrointestinal tract, are partially killed in the stomach. The rest of bacteria appears in the intestine and adheres to mucosal cells. Microbial intracellular invasion is promoted by salmonella ***needle complex***.

When injected into enterocytes, ***SPI-1 effector proteins*** stimulate cytoskeleton remodelling and next membrane folding. It leads to ***engulfment of attached bacteria*** and their entry into epithelial cells by macropinocytosis. Other SPI-1 proteins activate membrane channels of epithelial cells resulting in chloride excretion and ***diarrhea***.

In parallel with infection of intestinal epithelium, salmonellae spread into the lymphatic follicles and Peyer's patches. Microbial cells have multiple mechanisms of survival within phagocytes.

Certain SPI-1 proteins *activate caspase-1* that stimulates ***production of proinflammatory cytokines*** and eventually triggers ***phagocyte apoptosis***. Inflammatory cytokines damage the intestinal tissues.

Effector proteins, associated with ***SPI-2***, play even more powerful role in microbial protection against phagocytosis. Once captured by phagocyte,

salmonellae long time survive within phagolysosome. It is related with ***SPI-2 effector proteins*** that *block* the ***enzymes of respiratory burst*** thereby inhibiting microbial digestion.

Infected phagocytes spread salmonellae throughout the body resulting in ***systemic*** character of infection. Thus, the presence of ***genes of SPI-2*** strongly predisposes to the ***generalized salmonellosis***.

Bacterial transition across the intestinal wall leads to their appearance in the bloodstream with subsequent microbial dissemination. Salmonellae affect lymph nodes, spleen, liver, bone marrow, etc. Microbial death results in massive ***LPS endotoxin release***. It provokes ***systemic inflammatory response*** and ***vascular damage*** that causes cardiovascular and CNS disorders.

At the end of the first week of disease high fever, headache ("status typhosus"), myalgia and roseolar skin rashes arise. These symptoms are followed by hepatosplenomegalia. To the third week salmonellae accumulate within bile ducts and gallbladder and then re-enter the intestine. Multiple inflammatory reactions induced by microbial cells cause intestinal lesions and necrosis of lymphoid tissue. At this time bacteria are intensively released from patient's intestine with feces. Also they are excreted with urine.

As the disease confers both cellular and humoral ***immunity***, the immune reactions ultimately eliminate bacteria promoting patient's recovery. The immunity is rather stable, but sometimes reinfections occur.

Nevertheless, appropriate conditions for salmonella survival especially within ***gallbladder*** maintain microbial persistence and often cause the development of carrier state. Expression of capsular ***Vi-Ag*** increases bacterial resistance to bile salts.

Long-term (sometimes – lifelong) salmonella carriers are proven to be the most frequent sources of S. Typhi infection.

Paratyphoid infections caused by S. Paratyphi A, S. Paratyphi B or S. Paratyphi C are characterized by similar but modest clinical course with favorable prognosis.

Salmonelloses pertain to large widespread group of diseases, caused by non-typhoidal salmonellae. They are transmitted by ***fecal-oral*** and ***contact*** roots.

The infected and sick ***animals*** are regarded as the main ***sources of infection***.

Incubation period is short – from 2-6 hours to 2-3 days.

The infectious dose is definitely higher than of enteric fever agents – about 10^6-10^8 microbial cells.

The disease usually evolves after ingestion of contaminated foodstuffs (poultry – about 50% of disease cases, also eggs, meet, dairy products, etc.) Various serovars of salmonellae (e.g., S. Enteritidis, S. Choleraesuis, S. Anatum, S. Derby, and many others) can cause these diseases. The symptoms of ***severe food poisoning*** (enterocolitis, fever, vomiting, diarrhea, collapses, etc.) can appear even in few hours after infection onset due to the large microbial load.

Endotoxin is released from destroyed bacterial cells. Toxin triggers ***inflammatory*** reactions and affects gastrointestinal tract and cardiovascular system. Some bacterial serovars (e.g. S. Enteritidis) express ***enterotoxin***, which causes profuse diarrhea.

Mild forms of diseases are assumed to be self-limited, but severe intoxications lead to generalization of infection with septicemia.

Another type of ***salmonellosis*** resulting in *systemic disease* (***septicemia***) can develop in newborns or in immunocompromised patients.

The disease is transmitted from human carriers or sick persons. It usually occurs as ***hospital-acquired infection***. Very often it is caused by *multiple antibiotic-resistant* strains of **S. Typhimurium** or S. Enteritidis and finally results in endotoxemia and ***septicemia***.

Systemic character of infection is largely related with bacterial pathogenicity island ***SPI-2*** encoding effector proteins that inhibit phagocytosis.

Salmonellosis with septicemia has serious prognosis and may be fatal especially in infants.

Post-salmonellosis ***immunity is weak***, short-term and low specific.

Laboratory Diagnosis of Enteric Typhoid Fever and Salmonelloses

Specimen collection for diagnosis of ***enteric typhoid fever*** depends on pathogenesis stage.

Hemoculture examination is repeated from the first week of the disease. Stool specimens are examined on the second week from enteric fever onset. Slightly later the urine can be taken for the investigation. Bone marrow culture is examined much more rarely.

For isolation of salmonellae from patient's blood ***hemoculture*** investigation is performed. About 10-15 ml of blood are inoculated into 100-150 ml of liquid selective medium, e.g. into bile broth.

After overnight incubation the material is planted onto a differential medium (McConkey agar, EMB agar). Salmonellae grow as ***lactose-***

negative colorless colonies. To isolate the pure culture of salmonellae the material from lactose-negative colonies is re-inoculated into slant agar with appropriate differential media. For instance, the growth in double sugar agar or ***Russel's*** medium (contains meat-peptone agar, 1% lactose, 0.1% glucose, and indicator) reveals the color change only within the butt of medium that ensues from glucose fermentation.

Isolated culture is identified by two-step slide ***agglutination test*** according to Kauffmann-White scheme. The serogroup is defined by specific O-antigen agglutination and microbial serovar is determined further by agglutination with specific anti-H serum.

The examination is accomplished by culture biochemical tests and phagotyping. The latter test is elaborated with large number of specific phages. Vi-I bacteriophage is regarded as universal and reacts with all cultures of S. Typhi bearing Vi-Ag.

In case of ***stool specimen examination*** the material is inoculated into bile broth, selenite broth, tetrathionate broth or another selective media to inhibit concomitant flora. Also it may be planted on ***bismuth sulfite*** medium (Wilson-Blair agar) resulting in black salmonella colonies. Further investigation is similar with hemoculture isolation.

For ***serological diagnosis*** specific antibodies against microbial antigens are tested. Antibody titer arises at the end of the second week of the disease. Growth of specific antibodies is usually detected by ***indirect hemagglutination*** test or by ***tube dilution agglutination*** (***Widal's reaction***) with typhoid and paratyphoid A and B antigenic diagnosticums. Patient's serum is regarded as positive in titer of 1:200 and higher.

Typhoid patients with manifested disease demonstrate high titers of antibodies both to O- and H- microbial antigens. Convalescent or previously vaccinated individuals maintain the elevated level of H-antibodies for a long time.

For ***carrier state determination*** the indirect hemagglutination test with Vi-antigen erythrocyte diagnosticum is used. Serum of salmonella carriers contains anti-Vi antibodies in titers 1:40 and more.

For ***laboratory diagnosis of salmonelloses*** stool specimens, vomit, food remnants, animal organs, patient's blood, urine, etc. should be tested repeatedly.

The material is inoculated into bile broth, selenite or tetrathionate selective medium or onto bismuth sulfite agar. Laboratory investigation is similar with typho-paratyphoidal culture isolation and identification.

Genetic typing of salmonellae is performed by ***PCR***.

Treatment and Prophylaxis of Enteric Fever and Salmonelloses

Various ***antibiotics***, affecting gram-negative bacteria (primarily, third generation cephalosporins or fluoroquinolones) are administered to the patients with typho-parathyphoidal diseases and salmonella-caused septicemia. Most cases of food poisoning and enterocolitis in adults don't require antibiotic treatment but need adequate infusion therapy.

Salmonellae reveal marked ***multidrug antibiotic resistance***, which is conferred by number of R plasmids. The resistance is easily transmitted throughout microbial population, thus susceptibility testing and resistance monitoring are the valuable measures in disease control.

Specific ***prophylaxis*** of enteric typhoid fever requires further advances. Previously used killed vaccines are regarded today as inappropriate due to their short-term activity and side effects. Two vaccines are implicated now for practical use. Chemical polysaccharide vaccine is derived from capsular Vi antigen of S. Typhi. Another live attenuated vaccine of S. Typhi (Ty21a) strain is the result of chemical mutagenesis. Nevertheless, they create only relatively short-term protection that lasts several years. Genetically engineered and DNA vaccines, based on various recombinant S. Typhi strains are intensively designed now.

Non-specific prophylaxis includes the prevention of water and foodstuffs from microbial contamination with proper control of their sanitary state, maintenance of hygienic regimens and sanitary regulations especially in food handling. All foodstuffs prone to possible microbial contamination must be thoroughly cooked or sterilized. The patients and salmonella carriers should be timely identified and treated. The infection sites require intensive disinfection.

ENTEROPATHOGENIC YERSINIAE

Classification

Yersinia genus belongs to the family *Enterobacteriaceae* and contains more than 10 species. *Yersinia pestis*, a causative agent of plague is the most virulent among them. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* usually cause self-limiting diarrheal diseases and food

poisonings in humans generally termed as ***yersinioses***. However, enteropathogenic yersiniae, especially *Yersinia pseudotuberculosis*, sometimes produce persistent chronic infections that can disable patients for a long time.

Causative agents of yersinioses have multiple biotypes and serovars.

Structure and Properties of *Y. pseudotuberculosis* and *Y. enterocolitica*

Morphology

All yersiniae are similar to some extent with other enterobacteria: small polymorphic gram-negative rods that exhibit bipolar stain. They look like short chains or diplobacteria under microscopy. Enteric yersiniae carry peritrichous flagella with numerous pili.

Cultivation

Yersiniae readily grow on ordinary nutrient media. The best growth temperature for yersiniae is about 25°C. At these conditions bacteria render motility; temperature rise to 37°C leads to motility loss.

Microbial growth exhibits small convex semi-transparent polymorphic colonies with slight brownish pigmentation.

Various lactose-containing media (McConkey, or EMB agar) and several special media are used for yersiniae cultivation. During cultivation the bacteria produce ***lactose-negative*** translucent colonies. Colonies of yersiniae can dissociate into S- or R forms.

Biochemical properties

Yersiniae are facultatively anaerobic bacteria. They display marked biochemical diversity. These bacteria are not lactose-fermenting, but they can utilize many other carbohydrates (glucose, maltose, mannitol, dextrin, glycerin, etc.) with acid formation. *Y. enterocolitica* ferments sucrose, while *Y. pseudotuberculosis* not. According to carbohydrate fermentation, *Y. enterocolitica* is divided into 5 biotypes.

Yersiniae have weak proteolytic activity. As other enterobacteria, they are oxidase-negative, but catalase positive. Yersiniae produce urease and can reduce nitrates into nitrites.

Antigenic structure

Yersiniae possess flagellar ***H-antigen*** and several somatic cell wall antigens. *Y. pseudotuberculosis* contains ***S-*** and ***R-somatic*** antigens, *Y. enterocolitica* has somatic lipopolysaccharide ***O-antigen***.

More than 50 serovars are described within *Y. enterocolitica* species; serovars 03, 08, and 09 most frequently cause the disease in humans. *Y. pseudotuberculosis* has at least 6 distinct serovars; but serovar 01 is a predominant human pathogen.

Virulence factors

Yersiniae express different virulence factors. After microbial cell lysis ***lipopolysaccharide endotoxin*** is released, which exhibits pyrogenic, hemolytic and proinflammatory activity. Certain serovars of *Y. pseudotuberculosis* can produce ***enterotoxin***.

Bacterial adhesin ***invasin*** is encoded by genes of nucleoid.

Large number of virulence factors is encoded by mobile genetic elements.

A 72-kb ***virulence plasmid*** (***pYV*** – *plasmid of yersinia virulence*) is responsible for microbial adherence, invasiveness, intercellular spread, and ability to survive and propagate within host lymphoid tissues. This plasmid contains yersinia ***pathogenicity island*** or ***Yop virulon***.

Yop virulon encodes yersinia invasive ***effector proteins*** (***Yop proteins*** or *yersinia outer proteins*). Also it codes for structures of type III bacterial secretion system (***injectisome***, or ***needle complex***), composed of ***translocator proteins*** (so-called ***Ysc proteins***). Needle complex promotes microbial adherence to host epithelial or immune cells and delivers invasive Yop proteins into them. When injected, Yop molecules impair the dynamics of the cytoskeleton, allow microbial penetration and intracellular spread, thereby promoting further bacterial invasion. Yop proteins sharply diminish the production of proinflammatory cytokines by macrophages and other immune cells that maintain bacterial survival within lymphoid tissues.

Resistance

Yersiniae are rather stable in the environment. They can readily withstand cooling up to -25^0C. Bacteria survive for several months in water and some foodstuffs, contaminated by bacteria (e.g., in butter for 150 days and in water for 200 days). They stay viable and even propagate in milk, fruits and vegetables.

Yersiniae are sensitive to heating, desiccation and also susceptible to disinfectant actions.

Pathogenesis and Clinical Findings in Yersinioses

Yersiniae inhabit the intestinal tract of many animals (rodents, hares, foxes, cattles, etc.), in which they occasionally cause diseases. ***Rodents*** as common ***sources of infection*** discharge the bacteria into environment with feces and urine.

The disease is transmitted from animals by ***fecal-oral route*** predominantly through contaminated foodstuffs, especially raw vegetables, salads, etc.

Infectious dose of bacteria is ***rather high*** (up to 10^7-10^9 microbial cells). More often the disease affects infants and young children.

Incubation period lasts about 5-10 days.

Yersiniae enter the gastrointestinal tract and bind to enterocytes and intestinal M cells by means of needle complex and number of microbial adhesins (e.g., by ***invasin***). Yersinia virulon complex ensures microbial invasion and ***intracellular persistence***. The bacteria propagate within intestinal mucosa, affecting ileum and other parts of gut.

Then yersiniae spread into intestinal lymphoid tissues (lymph nodes and lymphatic follicles) and suppress cytokine synthesis by immune cells, thereby ***inhibiting phagocytosis*** and maintaining microbial survival. Bacterial propagation results in inflammation and ulceration of the intestine.

Inflammatory tissue damage and ***enterotoxin*** production elicit watery or bloody ***diarrhea***.

Yersiniosis, caused by *Y. enterocolitica* is characterized mainly by modest diarrheal syndrome and usually *self-limited*.

Y. pseudotuberculosis can produce more *severe disease* with a tendency to microbial spread and persistency. Further invasion of bacteria results in their appearance in mesenteric lymph nodes and next in bloodstream with microbial dissemination and endotoxemia.

Slow elimination of bacteria may cause *chronic disease*, which is manifested by infectious allergy and autoimmune reactions with arthritis, skin lesions (erythema nodosum), and inner organ dysfunction.

The ***acquired immunity*** is low specific and weak, thereby recurrent diseases occur.

Laboratory Diagnosis of Yersinioses

For ***laboratory diagnosis*** of yersinioses stool ***specimens***, vomit, blood, food remnants can be examined.

As the number of yersiniae in stool is often small, the sensitivity of ***cultural method*** can be enhanced by "*cold enrichment"*. In that case the stool specimen is placed into phosphate buffered saline, pH 7.6, and incubated at 4°C for 2-4 weeks. The majority of intestinal flora dies but yersiniae can grow. Similar result may be obtained by primary yersinia infection on mice.

Sub-culture is produced on Endo or MacConkey agar. Microbial isolate is further identified by its biochemical and antigenic properties.

For ***serological diagnosis*** the specific antibodies against microbial antigens are determined at the second week of disease by indirect hemagglutination test or ELISA. The assays are considered to be positive in titers 1:100-1:200.

Treatment and Prophylaxis of Yersinioses

Yersinioses are usually ***treated*** with antibiotics that affect gram-negative bacteria (third generation cephalosporins, fluoroquinolones, tetracyclines, or trimethoprim-sulfamethoxazole). Bacteria are resistant to ampicillin and first-generation cephalosporins.

Vaccines are currently ***not available*** for disease prophylaxis, thus all measures of sanitary control with prevention of food and water resources from microbial contamination should be maintained to limit disease spread.

Chapter 5

PATHOGENIC VIBRIOS – CAUSATIVE AGENTS OF CHOLERA. CAUSATIVE AGENT OF BOTULISM. *HELICOBACTER PYLORI*

CHOLERA VIBRIOS

The Discovery of Cholerae Causative Agent and the History of Cholera Pandemics

Cholera-like diseases were known from the times of antiquity. Nevertheless, the first registered epidemic outbreak of cholera emerged in India in 1817. It spread throughout the Indian sub-continent and finally was established as the first cholera pandemic in Asia.

In 1883 Robert Koch discovered the causative agent of cholera, later termed as classical *Vibrio cholerae* biotype (biovar). Later in 1906 another biovar, *El Tor vibrio* was isolated by E. Gotschlich on the Sinai Peninsula from the body of dead pilgrim.

The end of XIX and the beginning of XX century were hallmarked with six cholera pandemics. In 1923 the 6^{th} pandemic of cholera affected the continents of southern hemisphere, North America and Europe.

In 1961 the seventh pandemic started from Indonesia, then spread to India and the Middle East, appeared in Africa in the 1970s and finally achieved South America at 1990s.

The 5^{th} and 6^{th} pandemics of cholera were caused by *V. cholerae* serogroup O1 of the biotype "classical". Nevertheless, the 7^{th} pandemic was produced by serogroup O1 *V. cholerae* of biotype El Tor.

Serogroup conversion of *V. cholerae* gave rise to novel *V. cholerae* serogroup O139 in 1992. It provoked the emergence of a new large epidemic in Bangladesh and India. Multiple cholera cases caused by O139 strains are being registered now in Southeast Asia. Sometimes this is regarded as the start of putative eighth cholera pandemic. However, O1 El Tor isolates are also detected on these territories as well as in other parts of the world. For instance, the great epidemic of cholera in 2010 in Haiti was caused by El Tor biovar.

Classification

Cholera vibrios belong to the order *Vibrionales*, family *Vibrionaceae*, genus *Vibrio* and species *V. cholerae*. More than 200 serogroups of *Vibrio cholerae* were described but only the members of ***O1*** and recently discovered ***O139*** serogroup were proven to cause the epidemic disease. *Classical Vibrio cholerae* biotype and *El Tor* vibrio biotype pertain to O1 serogroup.

Representatives of *Vibrio cholerae* beyond the serogroups O1 or O139 are the accidental agents of moderate human diarrheal disorders being of lesser clinical relevance.

Another member of *Vibrio* genus *V. parahaemolyticus* may cause diarrhea in humans; in addition, *V. vulnificus* engenders some individual cases of human wound infections or septicemia.

Structure and Properties of Cholera Vibrios

Morphology

Cholera vibrios are ***comma-shaped gram-negative*** curved rods 2-4 μm long. In old cultures or on artificial media these bacteria occur in grains, straight rods, threads or spiral forms.

Vibrios are non-sporeforming bacteria. Both biotypes from O1 serogroup are lack of capsule. However, they synthesize exopolysaccharide that provides microbial biofilm formation. It confers also the resistance of vibrios to chlorines and bacteriophages. *V. cholerae* of O139 group as well as other vibrios can produce capsule.

Cholera vibrios are ***monotrichous*** motile microorganisms that usually carry one polar flagellum. Bacteria possess numerous pili responsible for microbial colonization. Among them are ***mannose-sensitive hemagglutinin*** that ensures vibrio adherence to the chitin of marine zooplankton, and ***toxin-coregulated pili*** (***TCP***), which promote microbial intestinal attachment as well as reception of ***enterotoxin-encoding bacteriophage CTXφ***.

Cultivation

Cholera vibrios are aerobic or facultatively anaerobic bacteria. They actively grow on basic nutrient media with increased salt concentration of 2-3% NaCl (*halotolerant bacteria*). The temperature range for culture is from 14 to 42°C with optimum at 37°C.

Vibrios can withstand alkaline pH, thereby they readily propagate at pH 8.0-9.5. Also they are resistant to bile salts.

In case of nutrient deprivation bacteria are capable of transforming into viable, but non-culturable organisms.

On solid nutrient media *V. cholerae* produce opaque, granular, smooth, round and convex dome-shaped colonies with a light-blue shine. In alkaline peptone broth vibrio cultures form a pellicle that contains agglomerated cholera vibrios.

Gelatin cultivation results in transparent granular colonies that resemble broken glass on a microscopy. The growth is followed by gelatin liquefaction.

Various enrichment and selective media (e.g. ***alkaline MPA*** or ***alkaline nutrient broth***) are applied for *V. cholerae* cultivation. On ***thiosulfate-citrate-bile-sucrose*** (***TCBS***) agar with indicator bromine thymol blue the bacteria produce yellow colonies.

Biochemical properties

Cholera vibrios have a broad spectrum of biochemical activity. All vibrios are ***oxidase-positive*** that discerns them from *Enterobacteriaceae* representatives. They ferment various substrates with acid end products (glucose, maltose, sucrose, mannose, mannitol, galactose, starch, glycerol and others).

B. Heiberg divided vibrios into 8 groups according to their biochemical activity. Both classical *Vibrio cholerae* biotype and *El Tor* vibrio pertain to 1 Heiberg's group and ferment sucrose and mannose whereas arabinose and lactose not.

Bacteria produce ammonia, indole, and reduce nitrates to nitrites. Also they coagulate serum and milk, and liquefy gelatin.

Cholera vibrios render variable hemolytic and hemagglutinating properties.

Antigenic structure

Different vibrios share a common flagellar heat-labile ***H antigen***.

Somatic lipopolysaccharide (LPS) heat-stable ***O-antigen*** is responsible for microbial antigenic specificity. More than 200 serogroups of vibrios were distinguished by O-antigen variations. It was mentioned above that classical *Vibrio cholerae* biotype and *Vibrio cholerae* biotype *El Tor* pertain to O1 serogroup. O1 antigen contains A, B and C antigenic variations. Thus, three main serotypes within O1 serogroup were established: Ogawa (AB), Inaba (AC), and Hikojima (ABC).

Vibrio cholerae O139 was proven to originate from *El Tor* vibrio. It was happened after acquisition of gene cluster encoding the synthesis of novel O139 LPS antigen, which thereby substituted initial O1 antigen of *El Tor*.

Virulence factors

Numerous adherence factors of cholera vibrios play substantial role in disease pathogenesis. Most important are ***toxin-coregulated pili*** (***TCP***). These pili are encoded by *Vibrio* pathogenicity island ***VPI***. Temperate bacteriophage *VPIφ* is assumed to deliver VPI genes into cholera vibrios.

TCP pili are responsible for microbial intestinal colonization. Moreover, TCP, expressed by cholera vibrio, act as specific receptors to bacteriophage CTXφ (***cholera toxin encoding phage***). CTXφ code for the production of ***cholerogen***-enterotoxin by initially non-toxigenic bacteria.

LPS of the cell wall of O1 serogroup bacteria and the capsule of O139 group strains accelerate microbial intestinal colonization. Also bacterial LPS renders ***endotoxin*** activity.

Exopolysaccharide of *V. cholerae* actively participate in ***biofilm*** formation. ***Capsule*** of O139 strains protects them from phagocytosis.

Potent ***enterotoxin-cholerogen*** is the ***major virulence factor*** of cholera vibrios. Cholera toxin is a heterodimer, composed of one A subunit in combination with five B subunits with total molecular mass of 84 kDa.

B-subunits of toxin bind to the intestinal cells via cell membrane ganglioside receptor. Subunit A is translocated through cytoplasmic membrane into intestinal epithelial cells, undergoes thiol-dependent activation and promotes ADP-ribosylation of cell G-proteins. This stimulates cellular adenylate cyclase resulting in great increase of cAMP concentration. The rise of intracellular cAMP concentration blocks active sodium chloride absorption and increases chloride and bicarbonate secretion. The latter results in passive water loss with development of ***massive diarrhea***. This is followed by marked decrease of intravascular volume, life-threatening hypoperfusion of critical organs and hypotension.

The cholera vibrios produce a number of ***invasive enzymes***, e.g. hyaluronidase, collagenase, fibrinolysin, lecithinase, neuraminidase, and various proteinases.

Resistance

Vibrios are the natural components of aquatic ecosystems. Colonization of zooplankton, plants, filamentous green algae, crustaceans and other marine inhabitants protect bacteria from unfavorable

environmental conditions. The *El Tor vibrio* biotype is characterized by relatively high resistance. It stays viable for more than 1 month in sea and river waters, up to 10 days in various foodstuffs, etc.

Cholera vibrios can live in feces for about a month; also they readily survive at low temperature.

Vibrios are sensitive to heating, UV light and desiccation (e.g, heating at 100°C immediately kills bacteria). Likewise, they are very susceptible to disinfectant treatment and acid exposure. Low concentrations of hydrochloric acid inactivate bacteria within one minute.

Pathogenesis and Clinical Findings in Cholera

Cholera vibrios inhabit the water of rivers, seas and oceans. Most environmental O1 strains are lack of cholerogen expression, but only ***toxigenic V. cholerae*** can cause the disease. It is considered that natural strains acquire the number of virulence genes from pathogenic microbial variants, and these events can occur both in external and gastrointestinal environment.

The emergence of virulent *V. cholerae* strain results from the cascade of ***horizontal gene transfers*** that eventually convert non-pathogenic aquatic bacterium into life-threatening human pathogen. Now it is generally assumed that non-toxigenic bacteria become virulent only after transduction with several temperate bacteriophages. First transduction event confers microbial cell to express ***toxin-coregulated pili*** (***TCP***) – receptors for ***cholera toxin-encoding phage CTX***φ. Next cell transduction with ***CTX***φ allows affected bacteria to produce cholera enterotoxin.

Moreover, phage transduction is supposed to be responsible for bacterial LPS structure changes. LPS change results in creation of new serologic variants of bacteria (e.g., vibrios of O139 serogroup), which escape from established human population immunity and provoke new large outbreaks of the infection.

Cholera is ***anthroponotic*** disease.

It is transmitted from sick persons and carriers by ***fecal-oral route*** with infected foods or water. The causative agent is also carried by flies, or can be transmitted by contact route through contaminated hands.

Short ***incubation period*** of disease lasts from several hours to 5-6 days.

After oral ingestion of contaminated water or food most of *V. cholerae* are killed by acidity of gastric juice. Thus, the ***infectious dose*** for cholera vibrios is rather high (in the range from 10^6 to 10^{11} microbial cells).

The rest of bacteria colonize the intestinal epithelial cells of small intestine, attach to the microvilli, and ultimately start to produce enterotoxin-***cholerogen***. Toxin expression is activated by gradual decrease of bile concentration along the small intestine.

Action of cholera enterotoxin leads to the development of disease symptoms. Patient's stools resemble "rice water," and contain many epithelial cells, mucus, and large number of vibrios. In severe cases profuse watery diarrhea and continuous vomit results in lowering of body's temperature, ***hypovolemic*** and ***hypotensive shock*** with lethal outcome within first 12 h of disease. The total fluid loss can achieve 20-30 l per day in adults. Without adequate compensatory infusion therapy the mortality rate exceeds 20%.

Abortive and mild disease forms are observed in majority of cases of *El Tor* vibrio cholera. Patient's carrier state rarely exceeds 1 month.

Post-infectious immunity is high-grade but of short duration. The immunity is both antibacterial and antitoxic; antitoxic antibodies confer most efficient protection against the disease.

Laboratory Diagnosis of Cholera

The ***specimens*** are collected from stool, vomit, autopsy material, water, foodstuffs, etc.

Microscopy is used as a preliminary test. The agglomerated gram-negative cholera vibrios resembling fish shoals appear in slide smears from stool.

Rapid diagnosis procedures include ***dark field microscopy*** of the stool specimens that reveal comma-like motile bacterial cells, and ***immunofluorescence assay***.

Identification of cholera causative agent is performed in several steps.

The specimens are inoculated into alkaline peptone water and alkaline agar. After short 6-hour incubation at 37°C thin biofilm of aggregated bacteria is formed. The biofilm material is gram-stained, tested for oxidase, and examined in slide agglutination test both with O1 and O139 antisera taken in titer 1/100.

If first alkaline broth cultivation result in scarce microbial growth, the material is inoculated again into alkaline peptone broth.

After primary examination alkaline broth culture is planted onto alkaline agar, or TCBS medium. TCBS growth reveals yellow vibrio colonies due to sucrose fermentation.

The vibrio culture is examined by repeat slide agglutination test and oxidase test. The latter should be positive for all vibrios. To obtain the pure culture the isolate is further planted on slant alkaline agar. Final identification of culture is made by agglutination reaction with O1 and O139 sera, biochemical tests (mannose, sucrose and arabinose fermentation), positive indole test, and by susceptibility to the number of specific phages.

Molecular genetic methods of vibrio typing are used in specialized reference centers for epidemiological studies.

Classical *V. cholerae* and *El Tor* vibrios can be distinguished by the number of tests: both biotypes are sensitive to specific bacteriophages; *El Tor* biotype is resistant to polymyxin B, it expresses hemolysin and produces acetoin with positive Voges-Proskauer test. Classical *V. cholerae* has opposite traits.

Treatment and Prophylaxis of Cholera

The ***urgent treatment*** of cholera is based mainly on ***infusion replacement therapy*** that compensates the loss of water and electrolytes. In case of adequate infusion the patient recovers from the disease. Different antibiotics, affecting gram-negative flora, can be used to facilitate convalescence. Usually oral tetracyclines are administered.

Antimicrobial chemoprophylaxis and vaccine prophylaxis may be used for disease prevention in family contact persons.

For ***specific prophylaxis*** phenol-killed vaccine and cholerogen toxoid are occasionally used now. Nevertheless, they confer only the short-term protection for 6-12 months in 50-80% of vaccinated individuals.

Elaboration of modern cholera vaccines is based on live microbial strains, but this work should account the possibility of attenuated vaccine bacteria to acquire virulence genes from environmental strains.

Non-specific prophylaxis of cholera includes the improvement of sanitation, prevention of water and foodstuffs pollution, protection of sources of water supply; proper hygienic and sanitary control measures and cholera surveillance. First cases of disease should be verified and carefully registered with subsequent isolation and hospitalization of all patients, observation and laboratory testing of all contact individuals, current and final disinfection in departments for cholera patients.

CLOSTRIDIUM BOTULINUM – CAUSATIVE AGENT OF BOTULISM

The History of Discovery

A causative agent of ***botulism*** (L. *botulus* – sausage, *botulism* – poisoning by sausage toxin) was firstly discovered and studied by E. van Ermengem in 1896. He isolated microbial pathogen both from intestine and spleen of patients, died from intoxication, and at the same time from food they had ingested (ham remnants).

Classification

Botulism causative agent belongs to the order *Clostridiales*, family *Clostridiaceae*, genus *Clostridium*, and species *C. botulinum*.

Structure and Properties of *C. botulinum*

Morphology

Clostridium botulinum is a large gram-positive rod up to 8 μm in length. It is a motile peritrichous bacterium with oval terminal or subterminal ***spore***. Sporeforming cell looks like ***tennis racket***.

Cultivation

The optimal temperature for microbial growth is within the range 30-40°C. These clostridia are readily cultivated in anaerobic conditions at pH 7.3-7.6. Culturing on sugar-blood agar in anaerobic jar reveals filamentous irregular hemolytic colonies. The growing anaerobic culture has the smell of rancid butter.

Cultivation in Kitt-Tarozzi medium results in homogenous turbidity followed by microbial precipitation.

Biochemical properties

Causative agents of botulism are ***obligate anaerobes***.

They ferment carbohydrates (glucose, maltose, glycerol and some others) with acid and gas end products. Mixed type of fermentation results in acetic, butyric, and lactic acid.

Botulism clostridia express marked proteolytic activity. They produce hydrogen sulfide, ammonia, and volatile amines. Also they are able to reduce nitrates to nitrites, liquefy gelatin and coagulate milk.

Antigenic structure

C. botulinum is divided into 8 serovars (A, B, $C_{1\alpha}$, $C_{2\beta}$, D, E, F and G) according to antigenic variations of microbial exotoxin. A, B, E, and F variants are found to be extremely toxic for humans.

Also bacteria possess flagellar H-antigen and somatic O-antigen similar in all botulism clostridia.

Virulence factors

C. botulinum produces the ***most poisonous neurotoxin*** known to date. One human lethal dose of dry botulinum toxin is about 0.1 ng/1 kg of body weight.

In anaerobic conditions clostridia start to secrete exotoxin especially after propagation in various foodstuffs (meat, fish, canned mushrooms and vegetables, etc.) Toxin production is inhibited in presence of 6-8% NaCl and in acidic conditions. Its activity is also neutralized by specific antibodies.

C. botulinum ***exotoxin*** is composed of A and B subunits. Subunit A is responsible for toxic activity, while B portion preserves the molecule from acid inactivation in stomach. Also it is resistant to digestive enzymes of gastrointestinal tract.

Once ingested, the toxin is absorbed in gut. It reaches the nervous system and ***inhibits the release of acetylcholine*** at cholinergic synapses, resulting in muscular ***paralysis***.

Botulinum neurotoxin is a ***Zn-containing metal protease*** that ***destroys synaptic proteins*** (e.g., vesicle-associated protein, synaptobrevin, cellubrevin and others) in cholinergic synapses of motor neurons.

Resistance

Heating at 90°C for 40 minutes or boiling for about 10 minutes irreversibly inactivates botulinum toxin. Heating at 80°C kills vegetative forms of clostridia within 30 minutes. The spores have strong resistance and remain viable in soil and dust for years. They can withstand boiling for up to 6 hours and even keep their viability in large pieces of meat after autoclaving for 15 minutes at 120°C.

Standard disinfectants, such us 5% phenol, inactivate the spores of botulism clostridia after exposure for 18-24 h.

Pathogenesis and Clinical Findings in Botulism

Spores of *C. botulinum* can be found in the intestine of animals, birds and fishes. They permanently discharge spores into surrounding environment with feces. The spores retain viability in the soil for a long time and can appear on the surface of vegetables and fruits with the soil dust.

Infected animals and fishes are regarded as the major ***sources of infection***.

Botulism is transmitted predominantly by ***fecal-oral route*** after ingestion of contaminated meat products, canned mushrooms, poultry, sausages, or vegetables, smoked and canned fish and many other products. These foodstuffs may contain germinated spores and various amounts of exotoxin, produced by viable microbial cells. Also botulinum toxin may enter the body through the wound surface.

Incubation period of the disease varies from several hours to 10 days and even more that depends mostly on amount of absorbed exotoxin.

After ingestion and intestinal absorption of exotoxin it appears in blood and invades central nervous system, muscular and other tissues. ***Toxin affects the neuronal nuclei*** of spinal cord and brain, neuromuscular junctions, cardiovascular system. Toxin binding is irreversible.

Anticholinergic action of toxin cause deep CNS disorders that result in dysphagia, vomiting, dry mouth, swallowing troubles, aphonia, dizziness, headache, diplopia, and eventual muscular weakness and ***paralysis***. Diaphragm paralysis can cause the lethal outcome. Mortality rate is very high (about 20-40%).

Rare but severe clinical condition is *infant botulism*, where the ingested spores germinate directly in baby's colon because of its poor colonization resistance, and the nascent clostridia begin to produce exotoxin.

Natural anti-toxic immunity is almost not created being of very low grade.

Recovery from botulism is followed by gradual restoration of activity of cholinergic synapses.

Laboratory Diagnosis of Botulism

In most cases the clinical findings of the disease are enough evident to make right diagnosis.

For ***laboratory diagnosis*** of botulism the ***samples*** of food remnants, vomit, blood and patient's stool are examined. Stomach contents and various corpse tissues (small and large intestine, brain, spinal cord) are used for post-mortem examination.

The presence of botulinum toxin in the specimens is confirmed by ***neutralization reaction*** in mice or guinea pigs, by ***ELISA***, or by indirect hemagglutination test with erythrocyte antitoxin diagnosticum.

For culture isolation the samples should be previously heated at 80°C for 20 min to inactivate non-sporeforming bacteria. They are next inoculated into Kitt-Tarozzi broth or other equivalent media and incubated in anaerobic conditions. The isolated culture is further tested for biochemical and ***toxigenic properties***. Culture toxin secretion is revealed by experimental mice infection. ***Toxin serotype identification*** is performed by ***neutralization reaction*** with antitoxin type-specific antibodies.

Toxigenicity of culture can be also confirmed by ***molecular genetic tests*** (e.g., ***PCR***).

Treatment and Prophylaxis of Botulism

Non-specific measures of patient detoxication (stomach lavage, adsorbent treatment, infusion therapy) can decrease the amount of absorbed toxin.

Urgent passive immunotherapy includes the repeat injections of high doses of horse-derived ***polyvalent botulinum antitoxic sera*** against A, B, C, and E serovars. Botulism toxoid is sometimes used to elicit specific antitoxic immunity in affected patients. The persons, suspected to use foodstuffs with botulinum toxin, are treated with polyvalent antitoxic sera in lower doses to prevent severe intoxication.

Non-specific prophylaxis includes the prevention of food contamination and the maintenance of established industrial sanitary conditions of meat, fish, caviar, or vegetable canning, and their proper storage. Home preservation, canning and storage of similar products can't provide their complete decontamination, thus it should be excluded from practical use.

HELICOBACTER PYLORI – THE AGENT OF CHRONIC GASTRITIS, GASTRIC OR DUODENAL ULCER

The History of Discovery

Single reports about the presence of spiral microorganisms in gastric mucosa were repeated several times still from the turn of XIX and XX century.

Nonetheless, only in 1982 Australian scientists physician Barry Marshall and pathologist Robin Warren isolated spiral bacteria from gastric tissue biopsy of patient with chronic gastritis. By the experiment of self-infection B. Marshall and colleague have proved for the first time the association between these bacteria and the development of chronic gastritis. Multiple next studies completely confirmed this association, as well as established new links of these microbial agents with gastric and duodenal ulcer, gastric cancer and certain cases of lymphatic tumors.

In 1989 the novel pathogen has acquired its final taxonomic name "*Helicobacter pylori*". And in 2005 B. Marshall and R. Warren were awarded Nobel Prize in Physiology or Medicine for their outstanding discovery.

Classification

The genus *Helicobacter* of the family *Helicobacteriacea* currently comprises more than 35 microbial species (*Helicobacter pylori, Helicobacter heilmannii, Helicobacter mustelae, Helicobacter felis* and many others). The main agent of human diseases is ***H. pylori***. Some relations with human pathology are reported for species *H. heilmannii*.

It is generally ascertained that *H. pylori* plays the substantial role in pathogenesis of acute and chronic gastritis, gastric and duodenal ulcer. Furthermore, helicobacter infection predisposes to the development of stomach cancer and gastric lymphoid tumor MALT lymphoma.

Structure and Properties of *H. pylori*

Morphology

Helicobacter pylori is a short or medium-size ***gram-negative*** bacterium of S-like spiral shapes. Microbial cells carry 2-6 flagella attached to one pole of bacterial body (***lophotrichate*** bacteria). They have no spore or capsule.

Cultivation

Helicobacters are ***highly fastidious agents*** propagating only in ***microaerophilic*** (5-7% O_2) and ***capnophilic*** (near 10% of CO_2) gaseous conditions; in standard aerobic or anaerobic surroundings the bacteria can't grow.

Also they have narrow temperature optimum for growth near 37°C, being completely inactivated at 25-28°C or above 41^0C.

H. pylori requires special and selective nutrient media with multiple growth factors. It can be cultured in *blood* or *serum* agar supplemented with broad spectrum antimicrobials (e.g., vancomycin, trimethoprim and amphotericin B) that inhibit the propagation of concomitant bacteria. Primary growth is evaluated in 5-7 days of culture.

Biochemical properties

Helicobacters are ***microaerophilic*** bacteria. They are oxidase and catalase positive; express multiple enzymes – phosphatase, phospholipase, hyaluronidase, proteases; produce H_2S, demonstrate remarkable ***urease*** activity.

These bacteria utilize amino acids as nutrients; from available carbohydrates they metabolize only glucose.

Antigenic structure

The bacteria possess somatic LPS-containing ***O-antigen***, flagellar ***H-antigen*** and superficial *outer membrane proteins* (***OMP***), which are type-specific.

Virulence factors

H. pylori produces the number of adhesins, aggressive enzymes and toxins.

The major role in pathogenesis of helicobacter infection belongs to microbial exotoxins – *cytotoxin* ***CagA*** (*cytotoxin-associated gene A*) and *vacuolating cytotoxin A* (***VacA***).

Cytotoxin ***CagA*** is present in most of the virulent strains of *H. pylori*. It is encoded by the same name pathogenicity island ***cag***. Besides CagA cytotoxin, this island codes for ***type IV secretion system*** (***T4SS***) of *Helicobacter pylori*.

Translocator proteins of T4SS deliver CagA toxin into gastric epithelial cells. The main pathogenic functions of ***CagA*** include the impairment of cellular metabolism and activation of cell-mediated inflammatory reactions.

Vacuolating cytotoxin A or ***VacA*** binds to membranes of gastric epithelial cells. It demonstrates pleiotropic pathological effects against gastric mucosal membrane.

For instance, VacA elicits the secretion of ***proinflammatory cytokines*** by leukocytes. Moreover, the molecules of VacA toxin create membrane pores allowing their own entry into epithelial cells. When entered into the cells, the molecules of VacA toxin trigger ***cell apoptosis*** or at least they cause profound ***degenerative changes in gastric mucosa*** (cell vacuolization and disruption of cellular tight junctions).

Helicobacter ***peptidoglycan*** also stimulates inflammatory reactions within stomach wall.

In addition, *H. pylori* intensively produces the number of ***aggression*** and ***invasion enzymes***.

High level of expression is essential for microbial ***urease*** that catalyzes urea decay. This leads to the production of exuberant amounts of ***ammonia*** that not only damage the mucosal tissues but also ***neutralize the acidity of gastric juice*** thus fostering microbial survival.

Hyaluronidase and microbial flagella stimulate bacterial ***invasion*** into submucous gastric layer.

Microbial ***phospholipases*** destroy the membranes of epithelial cells.

Siderophore proteins provide the bacteria with iron.

H. pylori demonstrates primary ***genetic resistance*** to sulfonamides, glycopeptides, polymyxins and amphotericin.

Resistance

Generally helicobacters are low-resistant bacteria taking into account the narrow temperature range (34-40^{o}C) of their growth and toxic action of atmospheric oxygen.

Nevertheless, there are some individual reports about helicobacter survival in dental plaque, saliva, vomits and gastric juice.

Pathogenesis and Clinical Findings of Diseases, Associated with *Helicobacter pylori* Infection

Helicobacter infection is regarded as one of the most common in human population. About 50% of humans are infected with *H. pylori* (25-40% in developed countries, where the people above the age of 50 prevail, and up to 80% of population in developing states with substantial part of young individuals).

Nevertheless, only 10-20% of *H. pylori* carriers finally develop gastric or duodenal ulcer; likewise, lifetime risk of stomach cancer among infected persons is about 1-2%.

Hence, the progression towards complicated helicobacter infection strongly depends on pathogen virulence, individual health state and lifestyle, nutritional habits, the safe use of certain groups of medicines like nonsteroidal anti-inflammatory drugs (NSAID), etc.

It has been established that *H. pylori* species is hallmarked with high genetic variability that originates from active lateral gene transfer. Up to 30% of bacterial genes are involved into infectious process. Thus, individual alterations of microbial virulence predispose to various manifestations of *H. pylori* infection.

The ***source*** of *H. pylori* ***infection*** – infected humans.

The ***routs of transmission*** are not completely elucidated yet. In most of cases the infection is transmitted ***orally*** by *fecal-oral mechanism* or by *direct contact*. *Iatrogenic* spread of infection via *contaminated endoscopic equipment* also can't be excluded.

When entered the stomach, most of bacteria settle in gastric antrum where the local pH of mucosal tissue is higher. Next they move towards duodenum. Active locomotion of microbial cells promotes their invasion into *submucous gastric layer*. Here they attach to membrane glycolipid receptors.

Urease of *H. pylori* metabolizes urea with ***ammonia*** release that neutralizes the acidity of gastric juice, supports long-time microbial survival and directly damages gastric mucosa.

The most virulent are helicobacter strains with parallel production of both bacterial cytotoxins - ***CagA*** и ***VacA***.

The protein apparatus of T4SS injects toxin *CagA* and the fragments of peptidoglycan into gastric epithelial cells.

CagA interferes in normal life cycle of epithelial cells; peptidoglycan fragments stimulate inflammatory response via activation of transcription factor NF-kB. Together with *VacA* toxin they promote the development of

acute gastritis and/or ***duodenitis***. This is followed by local hyperproduction of proinflammatory cytokines (IL-8 and others) that stimulates neutrophil and lymphocyte infiltration of stomach wall.

Incubation period of acute gastritis doesn't exceed several days.

Without proper management acute helicobacter gastritis has evident chances for transformation into ***chronic disease*** especially under the action of other predisposing factors (smoking, alcohol consumption, treatment with NSAIDs, etc.)

The next course of infection largely depends on predominant localization of inflammatory process.

If chronic gastritis affects mainly the ***pyloric part*** of the stomach, it leads to permanent hyperproduction of gastrin and HCl that finally results in development of ***ulcer*** of *duodenal* or *antral* localization.

If chronic helicobacter gastritis progresses into ***chronic pangastritis*** with damage of cardia, fundus and body of stomach, it causes the gradual but irreversible destruction of gastric epithelial cells. The production of hydrochloric acid declines resulting finally in *chronic* ***atrophic gastritis*** with achlorhydria.

Chronic atrophic gastritis is the significant risk factor of ***stomach cancer***. That's why helicobacter infection is regarded as ***biological carcinogen***.

The influence of helicobacter virulence factors on proliferation of immune cells may cause the emergence of rare cancer disease ***MALT lymphoma*** – the tumor originated from gastric lymphoid follicules.

Despite intensive activation of local cell-mediated ***immunity***, inflammatory response is unable to eliminate the infection resulting in *lifelong helicobacter carriage*.

Only efficient complex antimicrobial therapy results in *eradication* (complete removal) of helicobacter infection.

Laboratory Diagnosis of *Helicobacter pylori* Infection

As helicobacter infection is common among the individuals, specific laboratory examination is usually required for the cohort of patients with gastric and duodenal pathology.

Two groups of ***laboratory methods*** are used for detection of *H. pylori* – ***non-invasive*** and ***invasive tests***; the latter need gastric biopsy specimens.

Rapid *non-invasive* ***carbon urea breath test*** discovers urease activity of *H. pylori*. The test is convenient for mass screening of people attending medical offices and clinics.

When tested, the examined person drinks urea solution radioactively labeled with [^{14}C] or [^{13}C]. Under the action of microbial urease labeled CO_2 is released that is registered in expired air.

Other non-invasive tests include *determination of* ***Ags*** of *H. pylori* in feces by ***ELISA*** test and detection of microbial ***DNA*** by ***PCR***.

Invasive tests presume the examination of gastric ***biopsy specimens*** taken during endoscopy.

For instance, ***rapid urease test*** detects helicobacter urease in gastric biopsy by placement of the specimen into urea solution. The decay of urea is followed by ammonia accumulation that elevates pH of the medium and changes the color of indicator dye.

The most reliable test for direct detection of *H. pylori* in biopsy specimen is ***microscopy*** with ***histological*** *hematoxylin-eosin* ***staining*** or Warthin-Starry's ***silver stain*** that is more sensitive. Also luminescent stain can be used, e.g. with acridine orange dye. Typical morphology of bacteria is observed.

For ***isolation of microbial culture***, the tissue specimen is inoculated into special media supplemented with antibiotics and multiple growth factors. Incubation is performed in microaerophilic conditions (5-7% O_2) with increased concentration of CO_2 (5-10%). Primary growth should be assessed in 5-7 days.

The isolated culture is further examined by microscopy, biochemical testing (e.g., for oxidase and urease), serological and molecular genetic tests.

Serological diagnosis uses ***ELISA*** test for evaluation of specific *antibodies* (***Abs***) in patient serum directed against *H. pylori* Ags.

Treatment and Prophylaxis of *H. pylori* Infection

To prevent unfavorable consequences of *H. pylori* infection, complete ***eradication*** of this pathogen is required.

Recommended first-line antimicrobial treatment (so-called "*triple therapy"*) includes *proton pump inhibitor* (e.g., omeprazole) and two antibiotics *amoxicillin* and *clarithromycin*. The efficacy of this regimen is more than 85%.

Microbiological confirmation of eradication is performed after the end of treatment course.

In case of first-line treatment failure, quadruple therapy is used expanded with colloidal bismuth salts.

In the light of growing antimicrobial resistance of *H. pylori*, antibiotics of other groups can be administered – metronidazole, tetracycline, and fluoroquinolones.

Despite the high frequency of successful eradication, the cases of reinfection with *H. pylori* are common mainly due to the broad spread of this agent among human population.

Prophylaxis of *H. pylori* infection remains ***non-specific***. It is based on general measures for efficient sterilization of medical instruments, antisepsis and disinfection.

Various kinds of candidate vaccines against *H. pylori* are under clinical trials now.

Chapter 6

CAUSATIVE AGENTS OF BACTERIAL RESPIRATORY INFECTIONS: MENINGOCOCCI, *HAEMOPHILUS INFLUENZAE*, BORDETELLAE, LEGIONELLAE, AND MYCOPLASMAS

MENINGOCOCCI

The History of Discovery

The meningococcus *(Neisseria meningitidis)* was primary isolated from the cerebrospinal fluid of patients with meningitis and studied in details in 1887 by A. Weichselbaum.

Classification of Meningococci

Meningococci pertain to the family *Neisseriaceae*, genus *Neisseria*, and species *Neisseria meningitidis*. They are further subdivided according to their antigenic features into serogroups and serotypes.

Structure and Properties of Meningococci

Morphology

Meningococci are ***gram-negative***, *bean-shaped* pathogenic ***diplococci*** that similar to other gram-negative bacteria are surrounded by an outer membrane composed of lipids, outer membrane proteins (***OMPs***), and lipopolysaccharides. Pathogenic meningococci are enveloped by a polysaccharide ***capsule*** attached to this outer membrane.

Menigococci are non-sporeforming non-motile organisms. They possess multiple pili and fimbriae.

Cultivation

Meningococci are fastidious bacteria and can't grow on basic nutrient media.

They should be cultured on media with blood, serum or ascitic fluid (***ascitic agar***), better in atmosphere, supplemented with 5-10% CO_2 (*capnophilic* bacteria).

Optimum temperature for growth is 36-37°C. Bacteria can't grow at 22°C. After 48 h of cultivation on solid media they produce transparent, convex, glistening, and elevated small colonies without hemolysis. In serum broth turbidity and a precipitate at the bottom of the test tube appears.

Biochemical properties

Meningococci are ***aerobic*** or facultatively anaerobic bacteria. They produce oxidase and catalase.

Generally meningococci show poor biochemical activity – the bacteria utilize only glucose and maltose with acid formation being lack of proteolytic activity.

Antigenic structure

Meningococci carry multiple antigenic polysaccharides and proteins in their cell wall and capsule.

They demonstrate more ***genetic diversity*** than most of other pathogenic human bacteria. This is explained by horizontal intraspecies recombination and gene incorporation from closely related *Neisseria* species.

Because of this striking variability 13 serogroups by ***capsule antigens*** (A, B, C, D, Y, W-135, etc.) and 20 serotypes identifying outer membrane proteins (***OMP***) were defined.

On the ground of antigenic properties of lipopolysaccharide, termed ***lipooligosaccharide*** (***LOS***) because of its relatively short sugar chain, another 13 immunotypes were described. Further additional typing is possible according to the antigenic properties of immunoglobulin Al (IgAl) proteases and pili.

Virulence factors

Pili and outer membrane proteins are the major adhesins that contribute to meningococcal attachment to mucosal cells.

The most essential bacterial virulence factor for survival in the bloodstream is its ***polysaccharide capsule***, which protects bacteria against complement-mediated bacteriolysis and phagocytosis by neutrophils.

IgA proteases of meningococci break down human IgAs, thus impairing mucosal immunity.

Hyaluronidase and *neuraminidase* promote microbial invasion.

Disintegration of meningococci leads to the release of a highly toxic ***LOS***-based ***endotoxin***. Its liberation produces large amounts of proinflammatory cytokines such as tumor necrosis factor-alpha (α-TNF), IL-1, IL-6, IL-8, γ-interferon, and various colony-stimulating factors.

Unlike other endotoxins, meningococcal LOS can be actively secreted by bacteria within membrane microvesicles, and microbial cells retain their viability after LOS shedding.

Resistance

Meningococcus is the microbial agent of low stability – it is destroyed by drying in a few hours. By heating to a temperature of 60°C the bacterium is killed in 10 minutes and to 80°C in 2 minutes. When treated with 1% phenol, the culture becomes inactivated in 1 minute. Meningococci are very sensitive to low temperatures.

Pathogenesis and Clinical Findings in Meningococcal Infections

Meningococcal infections affect only humans (***anthroponotic disease***).

Meningococcal disease occurs worldwide. The bacteria from serogroups B and C cause the majority of infections in industrialized countries. Strains of serogroups A and, to a lesser extent, C dominate in third-world countries.

Meningococcal ***carriers*** are the predominant ***source of infection***. The causative agent is localized primarily in their nasopharynx. About 10% of adult population may become the carriers of meningococci through the lifetime.

Infants and children remain to be the most susceptible group for the disease.

The infection is transmitted by the ***air droplet route***.

Several forms of meningococcal infection exist: ***meningococcal carriage***, ***meningococcal nasopharyngitis***, ***meningitis***, and ***meningococcemia*** (including ***fulminant meningococcal sepsis***).

Meningococcal carriage and meningococcal nasopharyngitis are the predominant forms of infection, being most spread in population.

Nevertheless, some patients develop severe acute meningococcal disease: meningitis and meningococcemia. Meningococcal meningitis is regarded now as a form of systemic meningococcal disease, which is always followed by microbial dissemination.

Systemic meningococcal infection is the ***invasive disease***. It is occurred after exposure to a pathogenic strain and colonization of the naso-oropharyngeal mucosa, followed by microbial passage through mucosal tissues, and survival of meningococci in the bloodstream.

Damage of the nasopharyngeal ciliated epithelium may be the first step that provokes colonization. After primary adherence to CD46, further microbial attachment is promoted by interaction of outer membrane proteins (OMP) to CD66 receptors.

Microbial binding to CD66 on phagocytic and endothelial cells activates phagocytosis and cytokine production and stimulates the engulfment of meningococci by epithelial cells resulting in their transcellular passage.

Microbial entry into the bloodstream leads to bacterial dissemination and ***endotoxin release***. It triggers ***massive proinflammatory cytokine liberation*** that may cause toxic shock. High cytokine concentrations reflect the depth of shock. Extensive ***disseminated intravascular coagulation*** (***DIC***) and tissue damage are the most severe complications of meningococcal endotoxemia.

Incubation period lasts from ***several hours*** to several days, i.e., acute meningococcal disease is one of the most swift-progressing infections.

In some patients with low degrees of bacteremia, meningococci can be eliminated spontaneously.

Other patients demonstrate sudden attack of the disease with high fever 39-40^{o}C, vomiting, rigidity of the occipital muscles, severe headache, and hemorrhagic skin rashes. Involvement of the cranial nerves results from the increase of the intracranial pressure. A large number of neutrophils are found in the cerebrospinal fluid.

In case of meningitis the inflammatory response is localized predominantly in an extravascular compartment.

If meningococcal sepsis (meningococcemia) has abnormally high fatality rate (20-50% and even more), meningococcal meningitis develops lower rate of lethality (about 1-5%) and post-infectious neurological sequelae (in 10-20% of patients).

Immunity to meningococcal infection is associated with the presence of specific bactericidal complement-dependent antibodies in patient's serum. These antibodies arise in the course of infection. They can be type-specific and/or group-specific. Antimicrobial antibodies prevent the development of invasive disease. Recurring infections are not common.

Infants are generally protected from the infection for 3-5 months by passive immunity via IgG antibodies transferred from the mother.

Laboratory Diagnosis of Meningococcal Infections

Nasopharyngeal swabs and blood samples are taken for culture. ***Specimens*** of cerebrospinal fluid (***CSF***) and skin petechiac biopsy are taken for microscopy, culture, and microbial antigen detection.

Meningococcal ***antigens*** can be rapidly determined in CSF by ***precipitation*** or ***ELISA*** test.

Microbial ***DNA*** in CSF is detected by molecular genetic tests (***PCR***).

Microscopy of gram-stained slides with the samples of centrifuged cerebrospinal fluid detects typical *gram-negative bean-shaped diplococci* within polymorphonuclear leukocytes (incomplete phagocytosis) or extracellularly.

Cultivation of clinical specimens is performed in serum, ascitic or blood agar, supplemented with antibiotics, suppressing gram-positive microflora (vancomycin, amphotericin or ristomycin). After incubation for 48 h in aerobic atmosphere with 5-10% CO_2 pure cultures of meningococci can be recovered from CSF or blood.

The bacteria are further identified by carbohydrate fermentation and agglutination with group and type-specific sera.

Antibodies to meningococcal polysaccharides (***serological diagnosis***) can be measured by latex agglutination or ELISA. Test for antibodies is elaborated mainly in cases of unclear meningococcal infection.

Treatment and Prophylaxis of Meningococcal Infections

Taking into account the fulminant character of disseminated meningococcal infection, it is generally accepted that the therapy should never be delayed by diagnostic procedures, and ***antibiotics*** *are the cornerstone of* ***treatment***.

Beta-lactam antibiotics (penicillin G or third-generation cephalosporins) are the ***drugs of choice*** for treatment of meningococcal disease. Azalides or chloramphenicol can be used in allergic persons.

Treatment of shock includes fluid resuscitation, administration of glucocorticoids, transfusion of fresh-frozen plasma, mechanical lung ventilation if required.

For ***specific prophylaxis*** various ***polysaccharide chemical vaccines*** based on group A and C capsular antigens were developed.

Currently, a quadrivalent vaccine containing the antigens of serogroups A, C, W, and Y is available. Vaccination is highly effecient in the control of outbreaks and epidemics of meningococcal infection conferring the protective immunity at least for 2-3 years. However, vaccination doesn't affect carriers.

The major drawback of these vaccines is the absence of activity against group B meningococci. It has been found, that group B polysaccharide mimics the human neuronal cell adhesion molecules; therefore, the use of group B capsular antigen for immunization elevates the risk of autoimmune response.

Now experimental group B vaccines based on meningococcal outer membrane proteins are under the clinical trials.

HAEMOPHILUS INFLUENZAE AND OTHER RELATED BACTERIA

The History of Discovery

Hemophilic bacteria were primarily discovered in the early 1880s by R. Koch, who detected them in conjunctival exudate of patient with purulent conjunctivitis.

Some time later, M. Afanassiev in 1891 and R. Pfeiffer in 1892 isolated similar bacteria from patients in the course of influenza epidemic. As the result, for a long time these pathogens were regarded as the causative agents of influenza and therefore, acquired their own species name *Haemophilus influenzae*.

Classification

Hemophilic bacteria pertain to the family *Pasteurellaceae* and genus *Haemophilus*. This genus comprises more than 10 species of bacteria; some of them are seriously pathogenic for humans. ***Haemophilus influenzae*** species is the dominant human pathogen. The members of this species commonly cause respiratory infections, but in certain cases they may trigger severe invasive disorders, such as meningitis or septicemia.

Similar pathogenic activity is sporadically demonstrated by *H. parainfluenzae*, *H. haemolyticus* and *H. parahaemolyticus* species.

H. ducreyi causes *chancroid* or *soft chancre* – one of the bacterial sexually transmitted diseases (STD).

Genera *Aggregatibacter* and *Pasteurella* that pertain to the same microbial family also harbor human pathogenic representatives. For instance, *Aggregatibacter aprophilus* can be isolated in patients with bacterial endocarditis; one more agent *Aggregatibacter actinomycetemcomitans* is an agressive oral pathogen that participates in progression of periodontitis.

Structure and Properties of *Haemophilus influenzae*

Morphology

These organisms are small 0.3-1.0 μm ***gram-negative*** polymorphic coccobacteria. They are non-sporeforming, but produce ***capsule***.

Cultivation

Hemophilic bacteria are rather difficult for culture. As fastidious microorganisms, they need the number of auxiliary factors for efficient growth.

For instance, they require ***factor V*** (*nicotinamide adenine dinucleotide* or *NAD*) and ***factor X*** (*hemin*), which are commonly present in red blood cells. Thus, the optimal medium for them is ***chocolate agar***, where erythrocytes are lysed by heating. The bacteria grow better in presence of elevated concentrations of CO_2.

Also *H. influenzae* can be cultured on *blood* agar, but only nearby paper disc impregnated with V and X factors. Likewise, *H. influenzae* may grow together with satellite hemolytic bacteria (e.g., *S. aureus*) that liberate factors V and X from red blood cells.

The colonies of bacteria are small, convex, and glistening. Pathogenic *H. influenzae* render ***S***- or M (mucous) forms of colonies. Non-pathogenic strains usually produce R-forms.

Biochemical properties

The bacteria are facultative anaerobes with mixed type of metabolism. They produce catalase and oxidase.

H. influenzae ferment glucose. Some strains produce indole and metabolize urea. The latter reactions are used for biotyping of *H. influenzae*.

Biochemical differentiation of hemophils and related bacteria is based on the number of tests, presented in table 4.

Table 4

Differential tests for various Haemophilus species

Species	Growth factors		Catalase	Oxidase	β-galacto-sidase	Hemolysis	Glucose	Sucrose	Lactose	Mannose
	X or V	CO_2								
H. influenzae	X, V	+	+	+	–	–	+	–	–	–
H. haemolyticus	X, V	–	+	+	+	+	+	–	–	–
H. parainfluenzae	V	–	+	+	+	–	+	+	–	+
H. ducreyi	X	–	–	–	–	–	–	–	–	–
A. aphrophilus	X	+	–	–	+	–	+	+	+	+

Antigenic structure

The bacteria possess thermostable somatic *O-antigen* made of lipooligosaccharide (or ***LOS***) and superficial capsular polysaccharide *K-antigen*. Six basic serovars or ***types*** (a, b, c, d, e, and f) are recognized by capsular K-Ag. Non-capsulated strains are referred to as ***nontypable***.

Protein M-antigen is present in non-pathogenic strains,

Virulence factors

The major factor of bacterial virulence is ***capsule***. It shows adhesive properties and prevents bacteria from phagocytosis and complement activity.

Most of severe invasive infections are caused by capsular strains of *H. influenzae* ***type b*** (or ***Hib***).

Unlike other types, Hibs are covered with the capsule that contains *polyribosil ribitol phosphate* (***PRP***). All other capsulated *H. influenzae* have a hexose instead of pentose (ribose) in the structure of PRP.

PRP is a strong T-independent antigen.

The *lipooligosaccharide* (***LOS***) shows ***endotoxin*** activity.

Multiple *pili* play a role of the adhesins. They promote microbial attachment to epithelial cells.

IgA proteases of hemophilic bacteria destroy human IgAs thereby downgrading mucosal immunity.

Synthesis of *beta-lactamases* confers microbial resistance to certain β-lactam antibiotics.

Resistance

H. influenzae is enough sensitive to environmental factors being rapidly inactivated outside the body. However, in sputum and mucus it stays viable up to 18 h, on plastic surfaces – for 12 h.

Microbial cells are readily inactivated by all standard disinfectants (e.g., sodium hypochlorite, phenol, or formaldehyde).

Pathogenesis and Clinical Findings of Infections, Caused by *Haemophilus influenzae*

H. influenzae is solely human pathogen engendering various kinds of ***anthroponotic infections***. Nevertheless, hemophilic bacteria especially their nontypable strains are the normal inhabitants of human respiratory tract.

The decline of mucosal immunity of respiratory tract as well as airway damages predispose to active propagation of *H. influenzae*. They replicate extra- and intracellularly and may enter the bloodstream.

Sick persons and ***carriers*** of *H. influenzae* are the major ***sources of infection***.

The diseases are transmitted by ***air droplet route*** and, to lesser extent, by *contact* route.

Children under the age of 4-5 years are the most susceptible to *H. influenzae*.

The infections caused by *H. influenzae* are divided into two main groups – *non-invasive* and *invasive*.

Non-invasive diseases affect the epithelium of respiratory tract. Among them are acute *sinusitis*, acute *otitis media* and exacerbations of

chronic bronchitis. They result from the colonization of bronchial mucosa by *H. influenzae* after the impairment of mucociliary clearance. In most of cases they occur as the complications of primary respiratory infections, e.g., caused by viruses.

Invasive diseases are predominantly associated with *H. influenzae* of ***Hib*** type. They comprise the severe disorders with ***hematogenous spread*** – ***meningitis***, ***epiglottitis*** (acute inflammation of epiglottis), ***pneumonia***, and ***septicemia*** (***sepsis***).

The leading clinical forms of infections, caused by various types of *H. influenzae* are presented in Table 5.

Table 5
Infections caused by H. influenzae

Infections	**Groups of patients**	**Types of *H. influenzae***
Non-invasive		
Acute sinusitis, acute otitis media, bronchitis, conjunctivitis	All age groups	Nontypable serovars: >90%
Invasive		
Epiglottitis, meningitis, pneumonia, osteomyelitis, septic arthritits, cellulitis	Children under the age of 4 years – 90%; other children and adults – 10%	Hib – about 90%; Nontypable serovars – 10%; e and f serovars – 1%
Bacteremia and sepsis	Newborns, postpartum women	Nontypable serovars: >90%

The association of the most severe infections with *H. influenzae* type b (***Hib***) seems to be related with the expression of ***PRP capsule*** by these bacteria. It protects Hib from phagocytosis, opsonization, and complement lysis, ensuring microbial survival in the bloodstream.

Hib-associated ***meningitis*** has the mortality rate of 3-6% in affected children. The bacteria cause acute pyogenic damage of brain tissues resulted from the inflammatory action of microbial ***endotoxin***.

About 10-20% of children recovered from meningitis retain long-term and stable neurological complications, e.g., hearing loss.

Local invasive disease caused by *H. influenzae* is ***acute epiglottitis*** that may result in purulent necrosis of epiglottis with asphyxia of infant.

Newborns and postpartum women are under the risk of development of *H. influenzae* ***septicemia***, largely mediated by nontypable bacterial serovars.

In adults *H. influenzae* may cause pneumonia or bronchitis mainly as a complication of primary viral or bacterial respiratory infection.

Post-infectious ***immunity*** is stable. It is maintained predominantly by antibacterial antibodies. Babies and infants are passively protected with maternal antibodies for 2-3 months after birth.

Laboratory Diagnosis of *Haemophilus influenzae* Infections

Specimen collection for laboratory examination depends on the clinical form of the diasease. Initially sterile media are of the most clinical value – cerebrospinal fluid (CSF), blood, pleural exudate, pericardial or synovial fluids.

Microscopy with Gram stain of the sediments of centrifuged CSF reveals small gram-negative non-sporeforming coccobacteria.

Rapid detection of antigens of *H. influenzae* type b (Hib) in cerebrospinal fluid, blood or pleural exudate is achieved by latex agglutination or ***ELISA*** test.

Molecular typing of DNA of *H. influenzae* in clinical samples is performed by genetic tests (***PCR***).

For ***culture*** of CSF or other biological fluids the clinical materials should be managed immediately, or stored maximum 30 minutes at room temperature before culturing to prevent microbial autolysis.

CSF is primarily centrifuged before inoculation. Microbial antigens are detemined in supernatants by ELISA.

Sediments of CSF are cultured on chocolate agar, or blood agar with factors V and X in aerobic atmosphere with 5-10% CO_2.

The test of ***satellite cultures*** on blood agar can be applied as well. Here *H. influenzae* is inoculated along the streaks of satellite hemolytic bacteria (e.g., *S. aureus*) that release factors V and X from red blood cells. The growth of *H. influenzae* is possible only in close proximity to hemolytic satellite culture.

After assessment of microbial growth, the bacteria are further identified by the number of biochemical and serological tests. Identification of specific antigens allows to determine antigenic type of *H. influenzae*.

Treatment and Prophylaxis of Infections, Caused by *Haemophilus influenzae*

Invasive infections, associated with *H. influenzae* (meningitis, acute epiglottitis, or septicemia) require urgent ***antibiotic treatment***.

Taking into account possible resistance of *H. influenzae* to penicillins, mediated by production of *beta-lactamases*, ***third-generation cephalosporins*** (cefotaxime or ceftriaxone) are the drugs of choice for treatment of these diseases. Fluoroqunolones and macrolides (clarithromycin) can be administered as well.

For ***specific prophylaxis*** highly efficient ***polysaccharide chemical vaccines*** based on capsular antigen of *H. influenzae* type b (Hib) were developed. They are commonly used in combination with DPT vaccine (diphtheria, pertussis, tetanus vaccine) and vaccine against hepatitis B.

Infants are vaccinated four times in 3, 4, 5 and 18 months after birth.

Non-specific prophylaxis includes isolation and successful treatment of patients, prevention of carriage, improvement of sanitary conditions, proper disinfection.

PATHOGENIC BORDETELLAE: CAUSATIVE AGENTS OF WHOOPING COUGH

The History of Discovery

The causative agent of whooping cough *Bordetella pertussis* was discovered and isolated from patients in pure culture by J. Bordet and O. Gengou in 1906.

Classification

Bordetellae pertain to the family *Alcaligenaceae* and genus *Bordetella*. Main pathogen is *Bordetella pertussis*, causative agent of ***whooping cough***. *Bordetella parapertussis* cause similar milder disease. *Bordetella bronchiseptica* rarely produce human respiratory diseases (opportunistic pathogen).

Structure and Properties of Bordetellae

Morphology

The bacteria are small 0.5-1.0 μm ***gram-negative*** oval-shaped non-motile rods, except *Bordetella bronchiseptica*, which possesses polar flagella. They are non-sporeforming, *Bordetella pertussis* produce ***capsule***. Bacteria stain poorly with aniline dyes, the ends of bacterial body stain more intensively.

Cultivation

B. parapertussis and *B. bronchiseptica* can grow on basic nutrient media.

Isolation of *B. pertussis* requires enriched media. As additional growth factors amino acids cystein and methionin are applied.

Bordet-Gengou medium (***potato-blood-glycerol agar***) with penicillin or caseine-charcoal agar can be used. The plates are incubated at 35-37 °C for 3-7 days in a moist environment.

The colonies are small, convex, and glistening, resembling globules of mercury. They can dissociate into S- or R-forms. *B. bronchiseptica* synthesizes brownish pigment.

Biochemical properties

Bordetellae are ***obligate aerobes***.

B. pertussis shows minimal biochemical activity. The bacteria metabolize glucose with acid production. They are lack of proteolytic activity and urease, but produce catalase.

B. parapertussis and *B. bronchiseptica* are more active, producing urease, nitrate reductase, etc.

Some strains express hemolytic activity

Antigenic structure

The causative agents of whooping cough share a common thermostable *somatic O-antigen* and superficial capsular antigens.

At least 14 somatic antigenic variations have been identified in various *Bordetella* strains. Factor 7 is generic and common to all *Bordetella* bacteria; factor 1 is essential for *B. pertussis*, factor 14 – for *B. parapertussis*, and factor 12 for *B. bronchiseptica*.

Virulence factors

B. pertussis produces various toxic and aggressive substances.

Pili play a role in adherence of the bacteria to the ciliated epithelial cells of the upper respiratory tract.

Capsule of *B. pertussis* protects against phagocytosis and takes part in adhesion.

Most of virulence factors are governed by genetic ***bvg regulon*** (***bordetella virulence gene***).

Filamentous hemagglutinin mediates adhesion to ciliated epithelial cells.

Pertussis toxin (***exotoxin***) is the main virulence substance. It has typical A and B subunit structure and renders *ADP-ribosylating activity*, influencing cellular metabolism.

Also it stimulates lymphocytosis, sensitization to histamine, and enhances insulin secretion.

Adenylate cyclase toxin, ***dermonecrotic toxin*** and ***hemolysin*** are also regulated by *bvg* genes.

The ***tracheal cytotoxin*** inhibits DNA synthesis in ciliated cells.

The *lipopolysaccharide* of the cell wall may cause the damage of epithelial cells of upper respiratory tract.

Resistance

B. pertussis is sensitive to environmental factors. Nevertheless, it can withstand exposure to sunlight for about one hour. The bacterium is inactivated by heating at temperature of 56°C for 10-15 minutes. It is rapidly destroyed in solutions of conventional disinfectants (e.g., phenols or chlorines).

Pathogenesis and Clinical Findings in Whooping Cough

Whooping cough, caused by *B. pertussis*, is a severe infectious disease of childhood.

This ailment affects only humans (***anthroponosis***), being transmitted by ***air droplet route***.

The possible ***sources of infection*** are *patients* in the early catarrhal stage of disease and *carriers*. Communicability is high, ranging from 30% to 90%.

Bacteria attach to and propagate on the epithelial surface of the trachea and bronchi. The blood is not invaded. The bacteria liberate the toxins and substances that irritate epithelial cells, causing intensive coughing.

After an ***incubation period*** of about 2 weeks, the "*catarrhal stage*" develops, with mild coughing and sneezing.

During the "*paroxysmal*" stage, the cough becomes explosive. The stage lasts for another 4 or 6 weeks. Necrosis of parts of the epithelium and polymorphonuclear infiltration produces peribronchial inflammation and interstitial pneumonia.

Blood cell count reveals marked lymphocytosis.

Secondary infection by staphylococci or *H. influenzae* may easily cause bacterial pneumonia. Obstruction of the smaller bronchioles and diminished oxygenation of the blood can cause *convulsions in infants* with whooping cough.

The disease course is protracted and may last for 2-3 months in total; convalescence develops slowly.

Whooping cough confers *stable long-term* ***immunity***, but rare recurrent diseases in adults may be severe.

Bordetella parapertussis produce a disease similar to whooping cough. The infection is often subclinical. These bacteria usually have a silent copy of the pertussis toxin gene.

Bordetella bronchiseptica may cause so-called "kennel cough" in dogs. In rare cases it may be responsible for human respiratory infections especially in immunocompromised persons.

Laboratory Diagnosis of Whooping Cough

A *saline nasal wash* is the most preferable ***specimen***. Cough droplets obtained with "cough plate" method during patient's paroxysm or nasopharyngeal swabs are of lesser clinical relevance.

For ***rapid diagnosis*** of bacteria in the specimen immunofluorescent test is used.

For ***culture isolation*** the specimens are inoculated into Bordet-Gengou medium or casein-charcoal medium supplemented with antibiotics, inhibiting concomitant microflora.

After incubation on Bordet-Gengou medium the pure culture of bacteria is further identified by its morphological, cultural, biochemical tests. Antigenic properties are determined by slide agglutination test with specific antibodies.

Serological diagnosis is employed at the end of the second week of the disease. Antibodies against filamentous hemagglutinin and pertussis toxin are determined by ELISA.

PCR is the most sensitive method of pertussis diagnosis. Primers for both *B. pertussis* and *B. parapertussis* should be included.

If available, PCR test should replace both cultural method and serological testing.

Treatment and Prophylaxis of Whooping Cough

Treatment with antibiotics from *macrolide* or *azalide* groups during the catarrhal stage of the disease fosters the elimination of pathogens and may have prophylactic effect.

Treatment in paroxysmal stage demonstrates only low impact on the clinical course of the disease.

For ***specific prophylaxis*** effective ***inactivated pertussis vaccine*** is used. Administration of ***acellular vaccines*** based on *pertussis toxoid* or *filamentous hemagglutinin* is preferable in comparison with the whole cell vaccines because of greatly decreased side effects. Infants should obtain three doses of pertussis vaccine during the first year of life followed by repeat boosters for a total of five.

Pertussis vaccine is usually administered in combination with toxoids of diphtheria and tetanus (***DPT vaccine***).

LEGIONELLA PNEUMOPHILA – CAUSATIVE AGENT OF LEGIONNAIRES' DISEASE

The History of Discovery

First mass outbreak of legionellosis designated later as "Legionnaires' disease" was registered in Philadelphia in 1976 among the participants of convention of American Legion (US veterans' organization). This disease

manifested as severe pneumonia. The outbreak demonstrated high case-fatality ratio above 15% – from 4400 delegates attending the meeting 182 have become ill and 29 died.

The causative agent of this disease was discovered in 1977 by J.E. McDade and C.C. Shepard after its isolation from the lung tissue of patients died from pneumonia.

Classification

The bacteria pertain to the order *Legionellales* and family *Legionellaceae*. This family includes the single genus *Legionella.*

Near 60 legionella species are known to date. Despite more than 20 species are encountered as human pathogens in certain clinical conditions, *Legionella pneumophila* is responsible for more than 90% of cases of legionella-associated infections including the most severe clinical forms like Legionnaires' disease.

Structure and Properties of *Legionella pneumophila*

Morphology

The bacteria are small polymorphic ***gram-negative*** rods with tapered ends; coccobacteria and filamentous forms can be observed.

Microbial cells have no capsule or spore, albeit possess 1-3 polar flagella.

These bacteria are facultative intracellular parasites. In natural conditions they replicate inside water protozoa, e.g. amoebae cells.

Life cycle of *Legionella pneumophila* includes ***two*** basic ***phases*** – ***replicative*** and ***transmissive***; each represents distinct morphological forms of bacteria.

In ***replicative*** phase under nutrient-rich conditions the bacteria actively propagate *intracellularly* within *Legionella*-***containing vacuole*** (or ***LCV***). Microbial cells look like *non-flagellated* long rods, which are low-cytotoxic and ***low-virulent***.

When the conditions become worsened, *Legionella pneumophila* transforms into short thick *motile rods*, which are stress-resistant, cytotoxic and demonstrate ***enhanced virulence***. These bacteria leave the host cell being capable of infecting new cells (***transmissive*** phase).

If replicated inside free-living amoeba cells, transmissive phase results in almost dormant ***spore-like*** but motile and virulent ***mature infectious forms*** (MIFs). In case of prolonged stay in water they turn into *viable but non-culturable* (***VBNC***) morphological forms.

Cultivation

Legionella pneumophila grows in aerobic conditions in atmosphere, supplemented with 5% CO_2.

The bacterium needs special media for culture like *buffered charcoal yeast extract* (***BCYE***) *agar* with cystein, iron salts (ferric pyrophosphate) and antibiotics, or blood agar with various supplements. The optimum temperature for growth is 35-37^{o}C. In 3-5 days "opal-like" gray-white colonies appear. Sometimes they may produce brownish pigment.

Blood agar culture may show hemolysis. Some strains produce autofluorescence.

As facultative intracellular parasites, legionellae grow well in cell culture lines and yolk sac of chicken embryos.

Biochemical properties

Legionella pneumophila is ***aerobic*** bacterium that produces oxidase and catalase.

As primary source of carbon and energy the bacteria largely use amino acids (e.g., serine). To lesser extent the bacteria metabolize glucose.

L. pneumophila has no urease, but possesses the number of proteases. Microbial cells liquefy gelatin and slowly hydrolyze starch.

Antigenic structure

L. pneumophila are divided into at least 16 serogroups by their thermostable *somatic polysaccharide O-antigen*.

Nevertheless, about 85% of all cases of Legionnaires' disease are related with bacteria of serogroup 1.

Bacterial H-Ag is lack of diagnostic value.

Virulence factors

L. pneumophila has powerful ***systems of protein secretion*** that ensure the translocation of *virulent effector proteins* into the host cells.

The structures of ***type IV secretion system*** (***T4SS***) generally termed as ***translocon*** deliver almost 300 microbial ***effector proteins*** into eukaryotic cell. They govern all the process of bacterial habitation inside the host cells

– from microbial entry and its replication in legionella-containing vacuole up to bacterial egress and infection of new host cells.

These proteins account for microbial long-term survival within phagocytes inhibiting ***phagosome-lysosome fusion***.

Many bacterial effector proteins share evident similarity with proteins of eukaryotic host cells thus emphasizing the unique capacity of *L. pneumophila* to interkingdom ***gene exchange***.

Additional ***type II secretion system*** of legionellae (***LSP*** – *Legionella* secretion pathway) stimulates the secretion of virulent microbial enzymes.

Among them are numerous ***phospholipases A*** and ***C*** that destroy the membranes of cells. Also the bacteria produce metalloproteases, phosphatases and other enzymes.

Bacterial exotoxins ***legiolysin*** and ***cytolysin*** contribute to the membrane pore formation, lysis of host cells and hemolytic activity of legionellae.

Outer membrane proteins participate in adhesion. The *flagella* foster microbial entry into the cells.

When living outside the natural hosts, *L. pneumophila* indispensably creates tough ***biofilm***, firmly attached to the underlying surface. Within biofilm the bacteria remain highly protected against natural and artificial biocides.

Resistance

As the bacteria normally live in freshwater reservoirs, they are markedly resistant in watery environment. They stay viable for years in tap water, artificial systems of water supply, cooling towers, fountains, spa baths, etc.

Protozoans, harboring the bacteria, protect them from the action of biocides. Nevertheless, microbial cells are generally sensitive to conventional disinfectants (e.g., chlorine-containing substances, phenol, aldehydes, ethanol, etc.) For water disinfection *chloramine* and calcium hypochlorite are commonly used.

Pathogenesis and Clinical Findings in Legionelloses

Legionellae are broadly distributed in nature. They are normal habitants of freshwater sources, where they predominantly live ***inside the ciliated protozoa*** (like amoebae *Acanthamoeba* or *Naegleria*) or in slime

moulds. Dwelling in protozoan cells is beneficial for bacterial survival protecting them from harsh environmental influences and providing with nutrients.

Generally present in low amounts in natural freshwater habitats, *L. pneumophila* intensively colonizes ***human-made acquatic systems*** that operate in temperature range 25-55^{o}C and produce large amounts of water ***aerosol***. They are found in hot-water supplies, air-conditioning cooling towers, baths, shower-rooms, whirlpool and thermal spas, etc. The bacteria form poorly permeable ***biofilm*** on plastics and other artificial surfaces.

As the environmental conditions play a decisive role in microbial propagation and spread, the infections caused by *L. pneumophila* are regarded as typical ***sapronoses***.

Humans are occasionally infected with *L. pneumophila* being exposed to infected water aerosol. Overall, humans are the "dead ends" for legionellae replication.

There are two main forms of human *L. pneumophila* infections – *Legionnaires' disease* and *Pontiac fever*. The individuals with healthy immune status usually demonstrate self-limiting illness or remain asymptomatic.

Pontiac fever is relatively benign infection of upper respiratory tract with favorable prognosis.

By contrast, ***Legionnaires' disease*** is severe lung disorder manifesting like ***atypical pneumonia*** with serious prognosis and high fatality rate especially in cases of epidemic outbreaks.

It is ***opportunistic infection*** predominantly affecting males with chronic lung diseases, smokers, immunocompromised or elderly persons, cancer patients, etc.

The disease may arise as hospital outbreaks.

Transmission route for the infection – *airborne* via infected ***aerosol***. Human-to-human transmission is not observed.

Incubation period varies from 2 to 14 days.

When appeared in the airways, *L. pneumophila* is captured by alveolar macrophages and epithelial cells. The bacteria enter the macrophages by macropinocytosis or coiling phagocytosis, thereby making *Legionella-containing vacuole* (***LCV***) isolated from cytoplasm by membrane.

All this process is controlled by ***effector proteins*** of *type IV secretion system*. These proteins also ***inhibit phagosome-lysosome fusion***, thus preventing microbial digestion and vacuole acidification.

Inside LCV the bacteria come into replicative phase and propagate. When the nutrients are exhausted, they undergo transformation into motile

transmissive virulent forms. By the action of cytotoxins and enzymes legionellae penetrate vacuole, move into cytoplasm and finally leave the cell through the pores, created in cytoplasmic membrane. This leads to the destruction of respiratory epithelium and macrophages and stimulates inflammatory response.

Newly generated bacteria commence to infect neighbouring host cells leading to microbial dissemination.

Legionnaires' disease affects ***lower respiratory tract*** – terminal bronchioli and alveoli – resulting in severe lobar ***pneumonia***.

The disease has sharp onset with fever, chills and headache. This is followed by cough, tachypnea, and chest pain.

Necrosis of lung tissue may stimulate further microbial spread. It results in ***systemic infection*** and *septic shock* with lung hemorrhages, damage of gastrointestinal tract, kidneys and CNS.

Lethality in Legionnaires' disease strongly depends on initial patient's state, comorbidity and quality of treatment. Usually it falls into the range 8-25% but in case of hospital outbreaks it may exceed 50% in persons with immunosuppression.

Humoral and cellular post-infectious ***immunity*** is type-specific, protective and relatively stable.

Laboratory Diagnosis of Legionellosis

The ***specimens*** are taken from sputum, pleural exudate, blood, urine, samples of lung tissue on autopsy.

Rapid detection of bacteria is elaborated by ***immunofluorescence test***; microbial antigens are determined by ELISA.

DNA of *L. pneumophila* in clinical samples is detected by genetic tests (***PCR***).

Microbial ***culture isolation*** is performed in blood agar and buffered charcoal yeast extract (***BCYE***) agar with cystein, iron salts and antibiotics.

After incubation for 3-5 days characteristic "opal-like" gray-white colonies are determined. The growth on blood agar is followed by hemolysis.

Microscopy of culture reveals small polymorphic ***gram-negative rods***.

Identification of microbial serogroup is made by agglutination test – most of virulent *L. pneumophila* pertain to ***serogroup 1***.

Additional biochemical tests for utilization of amino acids, proteins and carbohydrates are elaborated.

Besides agar plating, *L. pneumophila* can be cultured in various cell lines (macrophage or epithelial cultures) and in laboratory animals (e.g., guinea pigs).

Serological testing is performed by *indirect immunofluorescence* and *ELISA*. The diagnostic titer of patients' antibodies in single immunofluorescence test is 1:128 and higher.

Serological testing can be also carried out with ***paired sera tests***, where ***fourfold rise*** in antibody titer should be observed,

Treatment and Prophylaxis of Legionellosis

Favorable prognosis of Legionnaires' disease strongly depends on timely administrated adequate ***antibiotic treatment***.

Macrolides and ***azalides*** (e.g. azithromycin and clarithromycin) as well as *respiratory fluoroquinolones* are the ***drugs of choice*** for legionellosis treatment.

Additional cure includes fluid resuscitation, administration of glucocorticoids, mechanical lung ventilation if required.

Prophylaxis of infection is ***non-specific***. Sanitary control measures should prevent microbial contamination of public and private water systems as well as exclude the possibility of hospital outbreaks of Legionnaires' disease.

A proper strategy of prevention of disease spread in health care settings comprises efficient disinfection of systems of water supply, air conditioning, and patient management; laboratory testing of patients with hospital-acquired pneumonia for legionellosis; epidemiological investigations of disease outbreaks with clarification of transmission routes.

High-efficacy measure resulting in eradication of *L. pneumophila* from artificial water systems is the increase of temperature of circulating water above 60^{o}C.

From commonly available disinfectants chloramine demonstrates elevated biocidal activity against legionellae.

PATHOGENIC MYCOPLASMAS: *M. PNEUMONIAE* – CAUSATIVE AGENT OF PNEUMONIA

The History of Discovery

First mycoplasma representatives – the causative agents of pleuropneumonia – were isolated by E. Nocard and E. Roux from the lungs of cattle.

In 1944 M. Eaton isolated a filterable agent from patient's sputum, which caused pneumonia in animals (cotton rats). Further investigations carried out by R. Chanok, L. Haifflik, and M. Borrel confirmed that the Eaton's agent belongs to the mycoplasmas.

Classification

Mycoplasmas pertain to the separate phylum *Tenericutes*, class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*, which includes two genera with pathogenic representatives: *Mycoplasma* and *Ureaplasma*. More than 200 mycoplasmal species are known to date.

In humans several species of mycoplasma have evident clinical relevance. The most virulent agent *Mycoplasma pneumoniae* causes pneumonia; also this bacterium is associated with joint and some other infections.

Ureaplasma urealyticum, *Ureaplasma parvum*, *Mycoplasma hominis*, and in certain cases *Mycoplasma genitalium* can cause human nongonococcal urethritis, especially in association with other bacteria.

Structure and Properties of Mycoplasmas

Morphology

Mycoplasmas are the smallest bacteria known being 125-250 nm in size. They pass through the filters with 0.45 μm pore size; hence, they are comparable to large viruses. Mycoplasmas are highly pleomorphic gram-negative bacteria (appear in rings, bacillary and spiral bodies, filaments, granules, etc.) because they ***have no cell wall***.

The cells of mycoplasmas are surrounded by thick triple-layered ***membrane*** containing large amounts of sterols (as the result, mycoplasmas require sterols for growth).

They are lack of capsule; ureaplasmas can carry flagella.

Cultivation

The optimum growth temperature for mycoplasmas is 36-37°C. Despite mycoplasmas are membrane parasites, they can grow on cell-free media that contain lipoproteins and sterols.

For instance, many strains of mycoplasmas can be cultured in heart infusion peptone broth with 2% agar (pH 7.8) supplemented with human ascitic fluid or animal serum (horse, rabbit), as well as on blood, serum or ascitic agar. Mycoplasmas are resistant to thallium acetate that is used for inhibition of concomitant bacteria.

After cultivation for 5-10 days or even more the round colonies of minimal sizes appear that have a granular surface and a dark center resembling "*fried eggs*".

Some strains can produce hemolysis.

In cell cultures as well as *in vivo* mycoplasmas grow predominantly at the cell surfaces being attached to cytoplasmic membranes of the cells. Mycoplasmas readily multiply in the chorioallantoic membrane of chicken embryos.

Biochemical properties

Mycoplasmas are facultative anaerobes or microaerophils. Overall, they have limited number of enzymes with reduced metabolism.

Many mycoplasmas ferment glucose as the source of energy with acid end products; some strains utilize arginine, ureaplasmas require urea.

Certain species are able to produce peroxides.

Mycoplasmas acquire sterols for their growth directly from cellular membranes (***membrane parasitism***).

Antigenic structure

Basic antigens of mycoplasmas are glycolipids and proteins with variable structure. Microbial enzymes also demonstrate antigenic properties.

Virulence factors

The whole number of mycoplasmal virulence factors is not well elucidated yet. Mycoplasmas carry various adherence structures: *interactive proteins*, *adhesins*, and *adherence-accessory proteins*, responsible for microbial attachment.

Adhesin P1 is the major virulence factor of *M. pneumoniae* that impairs the function of ciliated epithelium.

Some strains were shown to produce toxin-like substances and ***hemolysins***.

Bacteria can generate cytotoxic *hydrogen peroxide* and *superoxide radicals*.

Membrane compounds of mycoplasma play the role of *superantigens*.

Resistance

Mycoplasmas are very sensitive to the environmental influences. They are easily inactivated by heating, drying, sunlight, UV irradiation, and pH fluctuations. The bacteria are destroyed under the action of conventional disinfectants.

Pathogenesis and Clinical Findings in Mycoplasmal Pneumonia

The mycoplasmas appear to be rather host-specific, being contagious and virulent only for the specific host.

Thus, the ***source*** of micoplasmal pneumonia is the *sick person* and *mycoplasma carrier*.

The disease is transmitted by ***airborne route***.

Minimum ***infectious dose*** of bacteria to cause pneumonia is very low – about 100 microbial cells.

M. pneumoniae attachs to the membranes of ciliated and nonciliated epithelium of respiratory tract (***membrane parasitism***). During infection, the bacteria remain extracellularly.

Cytotoxic substances, free radicals and peroxides, microbial superantigens as well as immune complex-mediated cytolysis and cellular inflammation intensify the injury of respiratory epithelium mainly resulting in interstitial pneumonia.

The ***incubation period*** of the disease varies from 1 to 3 weeks. Usually mycoplasmal pneumonia has moderate manifestations with torpid course of infection.

The clinical spectrum of mycoplasmal pneumonia varies from asymptomatic infection to serious pneumonitis.

The onset of the disease is usually faint with fatigue, dry cough, subfebrile temperature or fever, and sore throat.

Initially the patient demonstrates only moderate illness. The physical signs of pulmonary inflammation are poorly determined but X-ray examination shows intensive lung involvement.

Resolution of pneumonitis and clinical improvement are observed in 2-4 weeks.

The presence of antibodies to *M. pneumoniae* is associated with resistance to infection. Cell-mediated immune reactions occur as well. The pneumonic process may be attributed in part to an immunologic response rather than only to infection by mycoplasmas.

After manifested form of the disease the specific humoral and cellular ***immunity*** lasts for 5-10 years. Mild and subclinical cases of micoplasmal infections confer only short-term and low-grade immune response.

Laboratory Diagnosis of Mycoplasmal Pneumonia

The diagnosis of pneumonia caused by *M. pneumoniae* can be largely made on the ground of clinical findings and X-rays.

Laboratory testing has to confirm the clinical diagnosis.

The basic clinical specimen is ***sputum***.

Rapid detection of bacteria is performed with immunofluorescence; microbial antigens are determined by ELISA.

The most sensitive test for the detection of microbial DNA is ***PCR***. It remains the method of choice for laboratory diagnosis of mycoplasmal infections.

Culture isolation is the long-term and cumbersome technique; thereby it is performed mainly in reference laboratories.

Serological diagnosis for mycoplasmal pneumonia is confirmed by ELISA in paired sera test. The ***fourfold increase*** of the titer of specific antibodies indicates ongoing infection.

Treatment and Prophylaxis of Mycoplasmal Pneumonia

As micoplasmas are totally lack of the cell wall, they are endowed with the intrinsic resistance to all β-lactams. Also they are resistant to sulfonamides because of inabilty to produce folic acid.

Azalides, macrolides, and tetracyclines are the drugs of choice for ***treatment*** of mycoplasma-associated infections.

None of specific vaccines are available now for clinical use.

Chapter 7
CAUSATIVE AGENTS OF BACTERIAL RESPIRATORY INFECTIONS: PATHOGENIC MYCOBACTERIA AND CORYNEBACTERIA

MYCOBACTERIUM TUBERCULOSIS – CAUSATIVE AGENT OF TUBERCULOSIS

The History of Discovery

Causative agent of tuberculosis (*Mycobacterium tuberculosis*) was first discovered and thoroughly investigated in 1882 by R. Koch.

R. Koch and coworkers created the experimental animal models of the disease that made possible to study pathogenesis and immunity in tuberculosis. In 1890 R. Koch obtained complex antigenic substance (tuberculin) from tubercle bacilli and tried to use it for tuberculosis treatment. This attempt appeared to be unsuccessful, but later tuberculin was applied for tuberculosis immunodiagnostics.

In 1919 A. Calmette and Ch. Guerin created live attenuated vaccine against tuberculosis. The strain ***BCG*** (or *bacillus Calmette-Guerin*) was introduced into medical practice, and it is used now for specific prophylaxis of the disease.

At the middle of XX century first efficient drugs for tuberculosis treatment were worked out (streptomycin in 1944, para-aminosalicylic acid in 1946, and isoniazid in 1952), thereby making possible the control of this severe disorder.

Classification of Pathogenic Mycobacteria

Mycobacteria pertain to the order *Actinomycetales*, family *Mycobacteriaceae*, and genus *Mycobacteria*. To date more than 130 mycobacterial species are known.

Mycobacterium tuberculosis is the predominant causative agent of ***tuberculosis*** in humans.

Mycobacterium bovis causes tuberculosis in cattle and much more seldom in humans (about 2-5% of cases).

Several other mycobacteria, e.g. *M. africanum* can rarely produce human tuberculosis infection.

Causative agents of ***mycobacterioses*** comprise more than 60 species. These bacteria exert severe opportunistic infections. The predominant pathogens here are *Mycobacterium avium-intracellulare, M. kansasii M. ulcerans* and others. They usually affect immunocompromised individuals, e.g. AIDS patients.

Mycobacterium leprae is the causative agent of ***leprosy***.

Many of mycobacteria are acid-resistant saprophytes, e.g. *M. smegmatis*.

Structure and Properties of Mycobacteria

Morphology

Most of mycobacteria are thin straight rods without capsule, spores and flagella.

Being highly pleomorphic, the bacteria can appear in granular, coccoid, thread-like, branching and filtering forms. The latter can pass through bacterial filters similar with mycoplasmas and viruses. Various microbial forms can be found ***intracellularly***.

Mycobacteria are considered to be gram-positive, albeit they are poorly stained by aniline dyes.

Microorganisms reveal striking acid and alkaline resistance (so-called "***acid-fastness***"). These abilities ensue from particular chemical composition of mycobacteria. They contain the great number of chemically inert lipids, phosphatides and waxes, usually termed as ***mycolic acids*** (various high molecular weight hydroxy fatty acids) in a complex with cell wall mucopeptides. Besides lipid fractions, bacteria include various proteins and polysaccharides.

Acid-fast mycobacteria stain red by basic phenol fuchsin in ***Ziehl-Neelsen stain***, whereas other bacteria are sensitive to sulfuric acid treatment and counterstain with methylene blue.

Also mycobacteria are successfully stained by fluorescent dyes, e.g. auramine.

Cultivation

Mycobacteria grow very slowly in aerobic conditions. It depends on long period of microbial replication (about 15 h for doubling). Growth is possible within temperature range from 24°C to 42°C with optimum at 37°C.

Various selective and special media are used for their cultivation. ***Lowenstein-Jensen medium*** contains agar, egg yolk, glycerol, potato extract, asparagine, milk, salts and malachite green to inhibit concomitant microflora. Composition of ***Finn medium*** is almost the same, but asparagine is substituted by additional number of salts.

Middlebrook semisynthetic agar is composed of oleic acid, albumin, vitamins and cofactors, various salts, catalase, glycerol, glucose, and malachite green.

Primary growth on solid media is observed in 3-6 weeks. *Mycobacterium tuberculosis* usually grows in warty dry colonies (***R forms***) with cream-colored "ivory" pigment.

Saprophytic mycobacteria proliferate more rapidly and appear in few days. They are able to produce orange or yellow pigment.

Different *broth media* support the cultivation of small amounts of mycobacteria. Liquid media growth reveals thin, brittle, wrinkled film resulted from microbial hydrophobic substances.

Pryce's microculture method on narrow glass slides is available for rapid cultivation of mycobacteria in citrate blood.

Rapid advanced cultural methods (e.g., ***BACTEC radiometric broth system***) allow swift identification of *M. tuberculosis* in minimal amounts.

Modern ***BACTEC radiometric system*** is composed of liquid medium supplemented with [^{14}C]-labeled palmitic acid. The medium also contains a number of antibiotics with broad spectrum of action to inhibit concomitant bacteria. During cultivation *M. tuberculosis* metabolizes palmitic acid with formation of radiolabeled carbon dioxide, which is further registered by radioactivity counter. By this method the infection is detected in 7-8 days of culturing.

Biochemical properties

Mycobacteria are ***aerobic*** microorganisms. *M. tuberculosis* produces a number of oxidation-reduction enzymes, including thermolabile catalase-peroxidase and superoxide dismutase. Also the bacteria express lecithinase, phosphatase, and urease.

M. tuberculosis can utilize various carbohydrates and proteins.

Antigenic structure

Lipids and phosphatides, encased in mycobacteria, are generally regarded as moderate antigens or haptens.

Nevertheless, in complex with mycobacterial proteins they elicit both cellular and humoral immune responses. *M. tuberculosis* infection induces cell-mediated reactions of ***delayed type hypersensitivity*** with chronic inflammation. Also mycobacterial antigens activate production of specific antibodies usually in low or moderate titers.

Tuberculin is a peculiar antigenic complex composed of various tuberculoproteins and wax fractions. It causes hypersensitivity reactions evaluated by ***tuberculin skin test*** (***TST***).

Virulence factors

Toxic substances of mycobacteria are closely associated with microbial body and mainly release after microbial decomposition.

For instance, ***mycolic acids*** render toxic effects against host cells and tissues.

Cell wall structures of glycolipid nature (mycosides, mannosides, etc.) participate in microbial adhesion and inhibit phagocytosis.

In addition, *M. tuberculosis* possesses highly specific ***type VII secretion system*** (***T7SS***) that promotes active secretion of micobacterial proteins across lipid-containing cell wall of mycobacteria.

T7SS is characteristic for virulent strains of *M. tuberculosis* and *M. bovis* being absent in vaccine BCG strain.

With the aid of T7SS *M. tuberculosis* expels some virulence effector proteins into the cytoplasm of infected cells (e.g., macrophages). Among them are ***CFP-10*** and ***ESAT-6***, which inhibit respiratory burst and secretion of proinflammatory cytokines by macrophages. Other proteins prevent the recognition of mycobacteria by Toll-like receptors of immune cells.

Microbial enzymes *catalase* and *superoxide dismutase* contribute to the inhibition of respiratory burst by macrophages.

Also *M. tuberculosis* carries so-called "***cord factor***" – potent toxic glycolipid fraction (***trehalose dimycolate***), which inhibits biological oxidation in the host cells and induces chronic immune inflammatory response with granuloma formation.

The production of cord factor is determined in ***Pryce's microculture method***, where virulent mycobacterial cells are grouped in tightly braced chains (or «*serpentine cords»*) visible under the microscopy with Ziehl-Neelsen stain.

Tuberculin is toxic for guinea pigs in course of experimental infection and elicits hypersensitivity in human infections, followed by tissue inflammation.

Mutations in genes, encoding bacterial enzymes catalase and RNA-polymerase confer the resistance of mycobacteria to basic tuberculosis drugs of the first line – isoniazid and rifampicin.

Resistance

Mycobacteria show high resistance in the environment. They remain viable in water for 1 year, in soil for 6 months, in the home dust and dried sputum for several months. Due to high lipid contents the bacteria can withstand the action of generally used disinfectants, thus ordinary disinfection regimens should be prolonged to inactivate mycobacteria. The most efficient are chlorine-containing chemical agents.

Mycobacteria are resistant to majority of antimicrobial drugs.

Nevertheless, heating at a temperature of 100°C readily kills all mycobacteria. Pasteurization inactivates *M. bovis* in dairy products, thereby preventing alimentary transmission of the disease.

Also mycobacteria are susceptible to sunlight and UV irradiation.

Epidemiology, Pathogenesis and Clinical Findings in Tuberculosis

Tuberculosis is one of the most important threats for human health at the beginning of XXI century. According to WHO data, annual total number of disease cases is about 9.6 million.

It is generally assumed that about one quarter of affected persons dies from tuberculosis and its consequences, and most of patients are young adults. Moreover, mortality rate in untreated or untreatable disease exceeds 50%. Thus, tuberculosis remains the leading infectious cause of human death resulting in approximately 1.4 million lethal cases every year.

Moreover, tuberculosis is the major ***AIDS-indicator disease*** in HIV-infected persons. It develops at least in 30-40% of HIV-infected individuals being the major cause of death of AIDS patients (25-40% of total AIDS lethality).

Finally, the uprising threat that faces public health nowadays is the rapid spread of ***multidrug resistant*** (***MDR***) and ***extensively drug-resistant*** (or ***XDR***) ***tuberculosis***. Now about 3.5% of tuberculosis cases worldwide are produced by MDR mycobacteria, and in certain world regions (African countries, several Chinese and Russia provinces, Baltic states, etc.) their

incidence greatly exceeds 10-20%. Therefore, the global spread of MDR tuberculosis is a problem of great medical and social importance.

Overall, tuberculosis is a ubiquitous disease that affects various living beings including animals, birds, and humans. It is generally ascertained that one-third of the world's human population (about 2 billion people) is infected with *M. tuberculosis*.

However, humans demonstrate high natural resistance to tubercle bacilli; therefore, ***tuberculosis remains the social disease*** that strongly correlates with poverty, adverse living and working conditions and general economic decline.

The main ***sources of infection*** are *persons with active tuberculosis*. Sick animals (cattle) can also spread the disease.

The infection is transmitted predominantly by the ***airborne*** (***air droplet***) ***route*** and more seldom by contact route. Ingestion of contaminated foodstuffs, usually milk, is also possible especially for *M. bovis* infection. Very seldom the disease can be transmitted by direct inoculation that may occur among health care workers.

Overcrowding, malnutrition and starvation, inaccessibility of medical care and other hard socioeconomic conditions, as well as suppression of patient's immune system (e.g., by HIV infection), are in the direct relationships with tuberculosis susceptibility and pathogen dissemination.

After primary penetration mycobacterial infection usually remains latent. Without prophylaxis about 5-10% of infected persons produce tuberculosis disease.

Inhaled mycobacteria are ingested by lung macrophages and transported to regional bronchial lymph nodes. *M. tuberculosis* survives within phagocytes, ***blocking phagolysosome fusion***. Cord-factor inhibits cell migration within the inflammatory focus.

Primary lung tuberculosis is characterized by acute exudative lesion affecting *lung acinary tissue* with subsequent rapid involvement of *lymphatic vessels* and *regional bronchial lymph nodes* (***primary tubercular complex***). Cell-mediated inflammation leads to formation of *tubercular granulomas* (***tubercles***) with caseous necrosis in their centers. Multinucleated giant cells, epithelioid cells, lymphocytes, macrophages and fibroblasts surround inflammation focus with mycobacteria.

Typical symptoms of progressive pulmonary disease include intoxication, fever, productive cough with ***hemoptysis***, enlargement of lymph nodes, and abnormal results of chest X-ray examination.

Cellular immune reactions restrict inflammation area and terminate the propagation of *M. tuberculosis*. Primary specific process is resolved with

connective tissue progression, fibrosis, and calcification. Remaining live mycobacteria come into dormant state and usually persist intracellularly within macrophages or epithelial cells lifelong.

In case of decreased immune resistance especially combined with large microbial load further spread of pathogen is possible via bronchi, lymphatic and blood vessels. ***Disseminated disease*** affects lungs (***miliary tuberculosis***) or leads to ***extrapulmonary tuberculosis*** (tuberculosis of eyes, intestine, kidneys, tubercular meningitis, etc). These forms are much more severe and may cause patient's death.

Reactivation of viable mycobacteria is possible due to many unfavorable external or internal stimuli, and easily affects immunocompromised persons (e.g., HIV patients).

Secondary tuberculosis is characterized by chronic tissue lesions (*tubercles, cavities with caseous necrosis*, etc.), followed by disseminated ***fibrosis***. Secondary lesions are very difficult in treatment showing no tendency to self-recovery.

Immunity in tuberculosis is predominantly ***cell-mediated*** and ***non-sterile***, maintained by viable mycobacteria. Macrophages, dendritic cells and Th1 cell subsets produce the vast number of proinflammatory cytokines (e.g., γ-interferon), thereby inhibiting microbial propagation. Antibodies are not proven to possess substantial antimicrobial activity.

As it was mentioned above, human population has high natural resistance to *M. tuberculosis*.

Laboratory Diagnosis of Tuberculosis

Patient's ***sputum***, lymph nodes puncture contents, urine, pleural or cerebrospinal fluid, etc. are used for bacteriological examination. Conventional methods comprise the acid-fast stain, culture, and biochemical tests for detecting and identifying *M. tuberculosis*.

However, rising spread of tuberculosis with high incidence of MDR mycobacteria has required new rapid laboratory tests for *M. tuberculosis* identification.

Microscopy of acid-fast bacilli is a valuable primary test for laboratory diagnosis of the disease. Tubercle bacilli stain red by fuchsin in ***Ziehl-Neelsen*** method due to the remarkable acid resistance of bacteria.

Fluorescent microscopy is more sensitive method than Ziehl-Neelsen technique. Mycobacteria easily stain with luminescent dyes (auramine or rhodamine).

In case of small amounts of pathogen the clinical sample is treated ***to enrich*** microbial content. The material is digested with a mucolytic agent (e.g., *N*-acetyl-L-cysteine) and treated with sodium hydroxide that kills acid-sensitive microorganisms. After centrifugation the smears from the sediment are prepared and stained.

Nevertheless, microscopy can reveal only 10^4-10^5 microbial cells.

For ***culture isolation*** both solid and liquid media may be used.

After sulfuric acid treatment specimens are usually inoculated into egg-based media (e.g., ***Lowenstein-Jensen agar***, ***Finn medium***, etc.) After long-term cultivation bacteria are identified by cultural, biochemical and virulent properties.

M. tuberculosis growth appears in 15-60 days resulting in typical dry colonies with "ivory"-colored pigment (***R forms***). Bacteria grow only at 37-38^{o}C, being unable to propagate in ordinary media or when treated with salicylates. *M. tuberculosis* carries thermolabile catalase, produces urease, and reduces nitrates into nitrites. Guinea pigs are very sensitive to *M. tuberculosis*.

Cord factor production is essential for *M. tuberculosis*. It is estimated by Pryce's microculture method using several narrow glass slides placed into citrate blood. After 4-5 days of cultivation slides are stained by Ziehl-Neelsen acid-fast stain. Cord factor elicits "serpentine cord"-like aggregations of microbial cells visible on microscopy.

Mycobacterium bovis grows within about 40 days. Bacterial growth renders smooth round colonies, or ***S forms***. Microbial cells produce thermolabile catalase, urease, but can't reduce nitrates into nitrites. *M. bovis* is highly virulent for rabbits.

Atypical mycobacteria (e.g. *Mycobacterium avium-intracellulare* complex, *M. kansasii*, *M. microti*, *M. ulcerans*, etc.) are virulent in ***S form***, can grow at 22-45^{o}C and in salicylate presence, produce orange pigment, carry thermostable catalase, being lack of cord factor and urease.

Acid-fast ***saprophytic mycobacteria*** (e.g., *M. smegmatis*) are non-virulent, microbial growth evolves within 3-4 days; bacteria propagate in ordinary media resulting in S-form colonies with orange pigment.

Rapid advanced cultural methods like ***BACTEC radiometric broth system*** greatly accelerate identification of *M. tuberculosis* taken in minimal amounts.

By this method the infection is detected in 7-8 days of culturing.

For rapid mycobacteria identification in clinical specimens ***polymerase chain reaction*** (***PCR***) with specific primers is used. This method is the most promising technique for express-detection of virulent mycobacteria.

Experimental animal infection has only a little worth for diagnosis. Similar, determination of specific antibodies against *M. tuberculosis* is also of limited value due to low specifity of serological reactions in tuberculosis.

Tuberculin skin test (***TST***) or ***Mantoux reaction*** evaluates delayed hypersensitivity in *M. tuberculosis* infection. ***Tuberculin*** is a multi-component antigenic complex of *M. tuberculosis*, composed of various tuberculoproteins and wax fractions. R. Koch obtained it as glycerol-based filtrated suspension of killed tubercle bacilli. He applied it for tuberculosis treatment but without evident success. Nevertheless, tuberculin was proven to be worthy for diagnostics of tuberculosis. It was further purified to derive protein fractions (***tuberculin PPD*** or ***purified protein derivative***).

After intracutaneous injection of a definite amount (usually 2 tuberculin units) of tuberculin PPD to the patient previously exposed to *M. tuberculosis*, the papule (induration and redness) appear, being maximal in 24-72 hours.

A positive TST indicates that the person has been infected with *M. tuberculosis*. Test explanation may be difficult, because previous BCG vaccination, specific chemotherapy, or host immune status can influence the reaction. However, test conversion from negative to positive implies recent infection and possible current activity of tuberculosis. A positive skin test assists in diagnosis, and it is also helpful for evaluation of tuberculosis treatment.

More advanced version of allergic skin test in tuberculosis is recently devised ***Diaskintest*** (***DST***). It uses recombinant proteins of *M. tuberculosis CFP-10* and *ESAT-6* as infectious mycobacterial allergens for intracutaneous injection. This test has serious advantages against TST as it is not influenced by primary BCG vaccination (BCG bacilli are lack of *CFP-10* and *ESAT-6* virulence proteins).

Blood lymphocyte culture tests like ***interferon-gamma release assay*** (***IGRA*** test) are also of rising value in immunological diagnosis of tuberculosis. They determine patient's lymphocyte sensitization to *M. tuberculosis*. The test is based on detection of γ-interferon release after the challenge of lymphocyte culture with specific micobacterial antigens.

Finally, adequate patient management in tuberculosis is impossible without rapid antibiotic susceptibility testing of isolated *M. tuberculosis*

culture. For these purpose both cultural and genetic methods are used. In latter case ***PCR*** (like in GeneXpert system) and hybridization techniques are employed to detect bacterial genes, conferring the resistance to antimicrobial drugs.

Specific Treatment and Prophylaxis of Tuberculosis

Treatment course of tuberculosis patient lasts from 6 to 12 months. Long duration of treatment period ensues from slow metabolism of tubercle bacilli as they tend to intracellular persistency and permanent evasion from host immune system.

Very short list of antimicrobial drugs was proven to be effective in tuberculosis treatment.

They pertain to the ***first line drugs***. Among them are ***isoniazid***, ***rifampicin***, ***pyrazinamide***, ***ethambutol***, and ***streptomycin***. Pyrazinamide is a sole antimycobacterial drug that can affect intracellular forms of *M. tuberculosis*.

Combined use of first line drugs for 6 months of so-called "***short chemotherapy course***", recommended by WHO, yields cure rates rather than 80-90% and prevents the emergence of drug resistance.

Second line preparations (e.g., fluoroquinolones, ethionamide, cycloserine, etc.) demonstrate lower efficacy, but often increased toxicity, being of more seldom use.

The treatment of MDR and XDR tuberculosis poses serious difficulties.

MDR bacteria are resistant to *isoniazid* and *rifampicin*, whereas XDR microbial cells are additionally resistant to *fluoroquinolones* and one more drug of the second line.

In these clinical cases the treatment course may last for 1.5-2 years.

The worsened situation with the highly limited list of efficient drugs against tuberculosis stimulated the design of novel antimycobacterial agents. Some of them are already introduced into clinical practice (e.g., *bedaquiline* and *delamanid*). They are predominantly administered in MDR and XDR tuberculosis.

Non-specific prophylaxis of the disease is achieved by isolation and adequate treatment of tuberculosis patients. Hospital disinfection is made by 5% carbolic acid or chlorine-containing disinfectants.

Vaccination with ***live attenuated BCG vaccine*** is used for ***specific prophylaxis*** of tuberculosis. Vaccine contains avirulent strain of *M. bovis*,

obtained by A. Calmette and Ch. Guerin after 13-year continuous bacterial passage through bile-containing media.

Newborn infants undergo primary vaccination at 3-5 day of life. Human immunization with BCG vaccine reduces the risk of tuberculosis in vaccinated persons by about 50%.

MYCOBACTERIUM LEPRAE

The history of discovery

Mycobacterium leprae, causative agent of ***leprosy***, was discovered in 1874 by Norwegian scientist G. Hansen.

Structure and Properties of *Mycobacterium leprae*

Morphology

M. leprae is similar with *M. tuberculosis* in many respects.

They are ***acid-fast*** gram-positive pleomorphic bacteria that mostly appear as long straight or curved rods 1-8 μm in length. Granular, branching and other forms also occur. These microorganisms don't produce spores or flagella. The pathogens are enwrapped into capsule-like layer made of glycolipids and mannosides.

M. leprae stain ***red by Ziehl-Neelsen*** method. They are determined intracellularly in tight bundles, resembling *packets of cigars*.

Cultivation

The bacteria are not adapted to grow in artificial nutrient media. They are ***obligate intracellular parasites***.

M. leprae can propagate after inoculation into mouse footpad within 25-30 days. The most suitable model for bacterial culture *in vivo* is the experimental infection of *armadillos*, which produce high bacillary lepromatous leprosy.

Biochemical properties

Biochemical properties of *M. leprae* are not fully investigated because of bacterial slow metabolism and absence of feasible methods for

culturing. The bacteria pertain to microaerophils. They have the reduced number of enzymes in comparison with *M. tuberculosis*.

M. leprae produces enzyme superoxide dismutase that protects it against phagocytosis.

Antigenic structure and virulence factors

Bacterial antigenic structure as well as virulence factor production is also not completely elucidated. *M. leprae* contains antigenic polysaccharides and numerous lipids, including leprosinic oxy fatty acid, wax leprozine, and various phosphatides.

Antigenic specificity of bacteria is related with *phenolic glycolipid fraction* ***PGL-1*** of microbial cell wall.

Toxic substances of bacteria are associated with microbial body and release upon its destruction.

Glycolipid ***capsule-like layer*** protect *M. leprae* against phagocytosis. The enzyme superoxide dismutase inhibits respiratory burst in phagocytes.

Phenolic glycolipid fraction ***PGL-1*** suppresses the activity of dendritic cells and T lymphocytes and takes part in *M. leprae* binding to Schwann cells of myelinated nerve fibers.

During infection bacteria cause the allergic sensitization of host with remarkable ***cellular immune suppression*** and ***demyelination*** of nerve tissue.

Resistance

M. leprae shows similar resistance with *M. tuberculosis* and stays viable in tissues of human corpse for more than a year. Nevertheless, free bacterial cells rapidly lose viability in the environment.

Pathogenesis and Clinical Findings in Leprosy

Leprosy is an ***anthroponotic*** torpid chronic disease.

Due to the active strategy of treatment, total number of patients with leprosy seriously decreased – from 805,000 persons in 1995 to about 175,000 affected individuals at the end of 2014 predominantly in Asia and Africa.

People usually develop the disease after ***extremely long incubation period*** lasting from 3-5 years to several decades.

Illness acquisition is possible only after ***close prolonged contact*** of a person with leprosy patient.

It is generally considered that disease progression is strongly related with individual genetic predisposition. Natural resistance to leprosy is common and may cover about 95% of human population. Genetic mechanisms of the resistance are not well-elucidated. Probably, they are associated with genes controlling cellular immune response (antigen recognition, processing and presentation, cytokine production, microbial cell killing, etc.)

The disease is transmitted via ***airborne*** or ***contact*** route through the nasopharynx epithelium or injured skin. Various fomites play a role of auxiliary vehicles in the disease transmission.

M. leprae can persist only within living cells. The disease may be latent all over the life. Bacteria slowly disseminate throughout the body and affect skin, nasopharynx, larynx, eyes, peripheral nerves and other tissues. Microbial active propagation is possible in conditions of suppression of cellular immunity with inefficacy of phagocytosis.

It is considered that *M. leprae* persist predominantly within demyelinated nervous tissue, where the bacteria are able to maintain favorable conditions for their survival.

Within epineurium the bacteria target myelinating Schwann cells and macrophages and propagate. As the result, ***chronic granulomatous inflammation*** arises resulting in direct injury of peripheral nerves with their ***demyelination***.

Erythematous painless lesions with nodular infiltration appear in the skin. The damage of nerves is followed by paresthesia and polyneuritis. Trophic disorders lead to deep tissue lesions resulting in bone resorption. Sometimes it might be followed by phalanx self-amputations.

Three main clinical forms of leprosy are observed: ***lepromatous***, ***tuberculoid***, and ***undifferentiated***.

WHO distinguishes ***multibacillary leprosy*** and ***paucibacillary leprosy***.

Lepromatous type of disease is characterized by malignant course of infection with active microbial propagation within myelinated nerve fibers that results in severe tissue lesions and neurologic disorders.

The disease progression rests on the activation of suppressor immune cells and T helper 2 subsets. Together with deleterious effects of *M. leprae* itself (e.g., by the action of phenolic glycolipid ***PGL-1***) it strongly inhibits cell-mediated immune response. This abrogates limitations for microbial growth.

M. leprae in large amounts are determined in the sites of infection. Therefore, this clinical condition corresponds to ***multibacillary leprosy***.

The allergic skin test with ***lepromin*** (boiled extract of lepromatous node) is negative in this situation due to deep immune suppression.

Tuberculoid type of the disease develops benign course with favorable prognosis. Skin lesions and peripheral nerves are involved in the process but only few or lack of bacteria can be found there (***paucibacillary leprosy***).

Cell-mediated immunity is capable of controlling tuberculoid disease as ***T helper 1*** *cells remain active*. They stimulate macrophages and dendritic cells that is followed by sufficient production of proinflammatory cytokines (IL-1, IL-12, IL-18, α-TNF, γ-interferon). The ongoing reactions of delayed hypersensitivity tackle the infection.

Lepromin test is positive in this clinical condition.

Undifferentiated type is usually related with the intermediate stage of the disease that may result in leprosy progression.

Laboratory Diagnosis of Leprosy

Specimens from scrapings of nasal mucosa, skin lesions, lepromatous nodes and lymph node biopsies, patient's sputum and ulcer discharges are used for examination.

Microscopy is the basic method for laboratory diagnosis of leprosy. The slides are stained with ***Ziehl-Neelsen*** method. Intracellular bundles of acid-fast bacilli are observed.

The detection of *M. leprae* is also performed by ***immunofluorescence***.

The advanced molecular tests for laboratory diagnosis of leprosy are based on ***PCR*** that detects microbial DNA.

Allergic skin test with lepromin (*Mitsuda reaction*) is useful to distinguish lepromatous or tuberculoid type of the disease.

Serological testing is of limited value due to the moderate titers of specific antibodies. Antibodies against PGL-1 are determined by ELISA.

Specific Treatment and Prophylaxis of Leprosy

Recently WHO announced Global Leprosy Strategy 2016–2020 under the common motto "Accelerating towards a leprosy-free world". It has been stated that evident successes in leprosy management are essentially related with the availability of efficient disease ***treatment***.

Now ***MDT*** (or ***multidrug therapy***) regimen is commonly used. It presumes the administration of sulfone drug dapson, antimycobacterial agent rifampicin and clofazimine. The course of therapy for multibacillary leprosy lasts for 12 months to provide complete elimination of pathogens.

Prophylaxis is only ***non-specific***, though numerous attempts of BCG vaccination were performed with contradictory results. Leprosy patients, who are active producers of mycobacteria, should be isolated and treated until complete clinical recovery. The healthy children need to be separated from sick parents and, if necessary, treated with antimicrobial drugs for disease chemoprophylaxis.

CORYNEBACTERIUM DIPHTHERIA

The History of Discovery

Causative agent of diphtheria, *Corynebacterium diphtheria*, was discovered by E. Klebs in 1883. F. Loeffler isolated it in pure culture in 1884. E. Roux and A. Yersin first derived the main virulence factor of *Corynebacterium diphtheria*, diphtheria exotoxin, in 1888. Corresponding antitoxin antibodies were obtained by E. Behring and S. Kitasato in 1890. Finally, G. Ramon created first biological product for specific prophylaxis of diphtheria, diphtheria toxoid, in 1923.

Classification

Corynebacterium diphtheria pertains to family *Corynebacteriaceae*, genus *Corynebacterium*. It accounts for extremely dangerous toxinemic infection – ***diphtheria***. Closely related species *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* can carry *tox* gene that encodes diphtheria toxin production, thereby they can also exert the disease in rare conditions.

Structure and Properties of *Corynebacterium diphtheria*

Morphology

Corynebacterium diphtheriae (Lat. *coryna* – club) is a straight or slightly curved polymorphic rod 1-8 μm in length. Under microscopy the groups of bacteria resemble letters X or V. Branched and thread-like forms as well as short coccobacterial forms may occur.

The rods of *C. diphtheriae* frequently display terminal club-shaped bulges with ***volutin granules***, stained blue by ***Neisser stain***. Volutin is the store of polyphosphates for microbial cells.

Granules of volutin are detected also by luminescent microscopy (e.g., they stain orange-red with coriphosphine dye).

Microbial cells are gram-positive. They are lack of spores or flagella, but may possess capsule.

Cultivation

The optimal temperature for microbial growth is about 37°C, and the bacteria can't propagate at temperatures below 15°C and above 45°C. Optimal medium pH is 7.2-7.6.

These organisms grow in media enriched with proteins (coagulated serum, blood agar, and serum agar) or in sugar broth. On ***Roux*** (coagulated horse serum) or ***Loeffler medium*** (three parts of bovine serum and one part of sugar broth) visible growth appear in 16-18 hours.

Now blood tellurite agar, containing blood and potassium tellurite (***Clauberg II medium***) and cystine-tellurite agar or ***Tinsdale medium*** are most often used for *C. diphtheriae* culture.

According to cultural and biological properties, various biovars of *C. diphtheriae* were defined: ***gravis***, ***mitis***, and ***intermedius***, which differ in a number of properties. Recently a new *C. diphtheriae* biovar ***belfanti*** was described.

Corynebacteria of the ***gravis*** biovar produce large rough (***R forms***) rosette-like black or grey colonies on tellurite agar. The bacteria ferment starch with acid end products and produce a pellicle in meat broth. They are usually highly toxic with marked invasive properties.

The colonies produced by corynebacteria of ***mitis*** biovar on tellurite agar are dark, smooth (***S forms***), and glistening. Starch is not fermented. Bacteria cause hemolysis of animal erythrocytes and produce diffuse turbidity in meat broth. Cultures of this biovar are usually less toxic and invasive than those of *gravis* biovar.

The bacteria of ***intermedius*** biovar are transitional. They produce small (***R-S forms***) black colonies on tellurite agar. Starch is not fermented. Growth in meat broth results in turbidity.

Newly discovered biovar ***belfanti*** is similar to other bacteria, but can't reduce nitrates into nitrites.

It was proven that *gravis* biovar is isolated in epidemic outbreaks of diphtheria, while *mitis* biovar appears in sporadic cases of the disease.

Biochemical properties

The causative agent of diphtheria is facultatively anaerobic bacterium. *C. diphtheriae* ferments glucose with acid formation, whereas galactose, maltose, starch, and glycerol fermentation is variable. Bacteria have no urease, produce no indole, and slowly produce hydrogen sulfide. They reduce nitrates to nitrites except biovar *belfanti*.

C. diphtheriae has the enzyme ***cystinase*** that is determined in ***Pizu test*** (serum agar media with cystine and lead acetate is blackened due to lead sulfide production). Conversely, diphtheria agents ***have no pyrazinamidase*** enzyme.

Production of cystinase and lack of pyrazinamidase distinguishes *C. diphtheriae* from other corynebacteria.

C. diphtheriae expresses the number of virulence enzymes – catalase, hyaluronidase, neuraminidase, and DNAse.

Antigenic structure

There are two major antigenic fractions in corynebacteria. Superficial heat-labile ***type-specific K-antigen*** is of protein nature.

Somatic group-specific lipopolysaccharide ***O-Ag*** is heat stable.

To date 57 serotypes of *C. diphtheriae* have been determined by agglutination reaction.

Virulence factors

All toxigenic *C. diphtheriae* express extremely poisonous ***exotoxin***.

Bacterial toxigenicity is under the control of ***phage genes***. When some nontoxigenic diphtheria strains are infected with bacteriophage transduced from toxigenic diphtheria agent, the offsprings of the exposed bacteria become lysogenic and toxigenic. Thus, acquisition of phage leads to toxigenicity (***lysogenic conversion***). The actual production of toxin usually occurs only after activation of the prophage within lysogenic *C. diphtheriae*.

In addition, toxin synthesis is governed by *transcriptional regulator* ***diphtheria toxin repressor*** (***DtxR***) encoded by nucleoid dtxR gene.

DtxR is iron-dependent transcriptional regulator. When the concentration of iron is sufficient, it blocks the expression of diphtheria toxin. And vice versa, low iron concentrations render DtxR repressor inactive, allowing the synthesis of exotoxin.

Diphtheria toxin is a heat-labile polypeptide with molecular weight 62,000. After inner thiol reduction the molecule is splitted into two fragments. Portion B is required for the transport of fragment A into the cell. Fragment A *inhibits peptide chain elongation* ***factor EF-2*** by its ADP-ribosylation.

Block of protein synthesis disrupts normal cellular functions. Abrupt termination of protein synthesis is responsible for the ***necrotizing*** and ***neurotoxic effects*** of diphtheria toxin. Pure diphtheria toxin may be lethal in extremely low dose of 40 ng.

Other virulence factors include adhesive pili and fimbria, invasive enzymes, hemolysins, and cord-factor.

Cord-factor of *C. diphtheriae* (trehalose dimycolate) damages mitochondria, affecting the processes of respiration and phosphorylation.

Resistance

C. diphtheriae are relatively resistant to various environmental factors. For instance, they survive for two months at room temperature. Corynebacteria remain viable in the membranes of diphtheria patients at least for 2 weeks, in water and milk – for 20 days. The bacteria are killed by a temperature of 60°C and by 1% phenol solution in 10 minutes

Pathogenesis and Clinical Findings in Diphtheria

Patients suffering from the disease and *carriers* are the main ***sources of infection*** in diphtheria.

The disease is communicated by ***airborne*** (air droplet or air-dust) route. Transmission by various objects or ***fomites*** (toys, books, towels, utensils, etc.) and foodstuffs (e.g., milk) contaminated with *C. diphtheriae* is also possible.

Exotoxin plays the principal role in the pathogenesis of diphtheria, ***blocking protein synthesis***.

It crosses the mucous membranes and causes the destruction of epithelium. The necrotic epithelium forms grayish "***pseudomembranes***"

over the tonsils, pharynx, or larynx. They are tightly bound to the affected tissues. Any attempt to remove the pseudomembrane results in bleeding. Pseudomembrane respiratory obstruction (***diphtheritic croup***) can cause patient suffocation. The regional lymph nodes in the neck enlarge, and there may be total neck edema. The diphtheria agents continue to produce toxin within the membranes.

Toxin absorption results in ***distant toxic action*** with tissue damage, particularly degeneration and necrosis in myocardium, liver, kidneys, and adrenals, sometimes accompanied by hemorrhages. The toxin also exerts nerve damage, resulting often in paralysis of the soft palate, eye muscles, or limbs.

The incidence of diphtheria of other organs (eyes, ears, skin or genital tract) is much seldom.

Post-infectious ***active immunity*** depends mainly on the ***antitoxin*** contents in the blood. However, a definite role of the antibacterial immunity, associated with phagocytosis, T cells, opsonization and complement-dependent microbial lysis is also significant. Therefore, the immune response produced by diphtheria infection is both antitoxic and antibacterial.

In general, diphtheria confers not very stable immunity, thus reinfection may occur up to 10% of cases.

Laboratory Diagnosis of Diphtheria

Swabs from the throat, nose, or other lesions as well as diphtheria pseudomembranes are tested as clinical ***specimens***.

Neisser-stained smears are examined and reveal typical corynebacteria with volutin granules. As rapid sensitive test, ***luminescent microscopy*** is used with coriphosphine staining that determines the presence of orange-stained volutin granules within microbial cells.

Nonetheless, diphtheria diagnosis is confirmed only in case of ***exotoxin detection*** in the clinical specimen or in isolated culture.

Rapid determination of diphtheria exotoxin in clinical samples is elaborated by ***ELISA***; identification of microbial ***tox-genes*** is performed by ***PCR***.

Overall, ***PCR*** is regarded as ***the most sensitive***, rapid and specific test for the confirmation of toxigenicity of *C. diphtheriae*.

When ***cultured***, the specimens are planted onto special media, e.g. Loeffler ***coagulated serum***, ***Clauberg II medium***, ***Tinsdale agar***, etc.

Primary growth is assessed on the Loeffler slant in 12-18 hours. In 36-48 hours the typical colonies on tellurite-containing media are observed.

The isolated culture is further identified by biochemical and antigenic tests and by phagotyping.

For the ***determination of toxigenicity*** of isolated cultures various ***neutralization tests*** are applied.

In case of animal ***experimental infection***, the material can be injected into 2 groups of animals (guinea pigs or mice), where one of them was passively protected with diphtheria antitoxin. The unprotected animals die in 2-3 days, whereas the immunized ones survive.

Plate ***immunoprecipitation*** or ***Elek's test*** is made as follows: a strip of filter paper saturated with antitoxin is placed onto serum agar plate. The cultures to be tested for toxigenicity are streaked across the plate at right angles to the filter paper. After 16-24 hour incubation the antitoxin diffusing from the paper strip yields the precipitation of toxin diffusing from toxigenic cultures. As the result, precipitation lines are determined between the strip and bacterial growth.

The toxigenicity of *C. diphtheriae* can be also shown by inoculation of bacteria into ***cell culture*** monolayers (e.g., Vero cell cultures). It is followed by evidenr ***cytopathic effect*** of the toxin with the destruction of cell monolayer.

Specific Treatment and Prophylaxis of Diphtheria

The ***specific treatment*** of diphtheria rests largely on the ***early administration*** of specific ***antitoxic antibodies*** that neutralize highly poisonous exotoxin of *C. diphtheriae*. Treatment with antibiotics that causes rapid suppression of toxin-producing bacteria is also helpful in the disease management.

Diphtheria antitoxin (***DAT***) is horse serum-derived biological product. It is obtained by the repeated immunizations of horses with purified and concentrated toxoid with subsequent purification.

Treatment with antitoxin is mandatory for patient's recovery. From 20,000 to 100,000 units are injected depending on disease severety.

Skin test should be made before antitoxin treatment to detect possible hypersensitivity to animal serum proteins.

Antimicrobial drugs (e.g., penicillin G, clarithromycin or azithromycin) inhibit the growth of diphtheria agents. As the result, they greatly diminish

toxin production. Antibiotics also help to eliminate coexistent pathogenic bacteria (e.g., streptococci) from the respiratory tracts of affected patients.

Specific prophylaxis is achieved by ***active immunization***. Usually ***DPT vaccine*** or combined ***tetanus-diphtheria toxoid*** are used.

It should be emphasized that diphtheria is regarded as the disease fully ***preventable by vaccination***.

Population (or "herd") immunity above 95% is regarded as sufficient to cease the disease contraction among the individuals.

All the children must receive the course of diphtheria toxoid immunization. It is afforded thrice at the first year of life starting from the age of 3 month. Subsequent boosters are injected in 9-12 months and then reproduced every 10 years.

Chapter 8

CAUSATIVE AGENTS OF SEXUALLY TRANSMITTED DISEASES

TREPONEMA PALLIDUM – CAUSATIVE AGENT OF SYPHILIS

The History of Discovery

Syphilis, the most notorious ***venereal*** or ***sexually transmitted disease*** (***STD***) is known for many ages. Until quite recently two main theories of syphilis origin existed: pre-Columbian, which supposed syphilis to emerge in ancient times in Central Africa with farther spread towards Europe and Asia, and Columbian one, relied upon syphilis epidemic rise in Europe after Columbus voyage.

According to multiple investigations of fossils the first theory was preferentially supported for a long time.

Nonetheless, in 2008-2011 the thorough phylogenetic analysis of evolution of various groups of treponemas elaborated by K. Harper and colleagues has proven the agent of syphilis to be phylogenetically youngest from all other treponemas. The most probably it has been developed from some non-venereal treponema subspecies and later caused epidemics of syphilis in Europe after the voyage of Columbus.

It is generally believed that syphilis was named by G. Fracastoro in 1530 after a mythical shepherd, Syphilus, described in his poem "Syphilis or the French Disease".

Only in 1905 F. Schaudinn and E. Hoffmann discovered *Treponema pallidum* to be the causative agent of syphilis. They revealed spirochetes in Giemsa-stained fluid smears from secondary syphilitic lesions. Year later A. Wassermann proposed complement fixation test for serological diagnosis of syphilis.

First chemical drugs for syphilis treatment were introduced into clinical practice still by P. Erlich (organic arsenical compound salvarsan). In 1943 J. Mahoney demonstrated the effectiveness of penicillin for syphilis therapy, and it remains to be the most preferable drug for disease treatment.

Classification of Pathogenic Treponemas

T. pallidum belongs to the order *Spirochaetales,* family *Spirochaetaceae,* and genus *Treponema*.

Pathogenic species *T. pallidum* has 3 subspecies and 1 closely related species:

T. pallidum subsp. *pallidum*, which causes ***venereal syphilis***;

T. pallidum subsp. *endemicum* that causes ***endemic syphilis*** or ***bejel***;

T. pallidum subsp. *pertenue* that produces ***yaws***;

T. carateum, the agent of ***pinta***.

These microbial pathogens are very similar; their DNA homology exceeds 95%.

Structure and Properties of *T. pallidum*

Morphology

T. pallidum are ***gram-negative*** thin ***corkscrew-shaped*** bacteria about 0.2 μm in diameter and 5 to 20 μm in length with 6-20 regular small coils with tapered ends. The cytoplasmic membrane lends treponemas a spiral shape.

Microbial body consists of an axial filament and cytoplasm wound spirally around the filament. Cytoplasmic membrane is covered by three-layer outer membrane. It covers basal bodies with attached 3-4 bacterial ***endoflagella*** or ***fibrils*** localized in periplasmic space. Endoflagella provide active variable motility of bacteria.

Treponemas are lack of producing spores or capsules. Old treponema cultures form cyst-like structures.

The bacteria stain ***pale-pink*** with ***Romanowsky-Giemsa*** method as they poorly stained with aniline dyes due to the large lipid contents. Treponemas can be detected by silver impregnation method, dark field and phase contrast microscopy.

Cultivation

T. pallidum are extremely fastidious ***microaerophilic*** bacteria that maintain viability in presence of 1-4% oxygen. They can’t propagate in ordinary media. When cultivated at 37°C on rich artificial media with ascitic fluid and brain tissue under anaerobic conditions they gradually lose their virulence (*cultural treponemas*).

Nevertheless, *T. pallidum* grows well and maintain virulence by animal inoculation, e.g., in rabbit testicular tissue (tissue treponemas). However, rabbit infectivity test is long lasting and requires from 3 to 6 months for cultivation.

Cultural and tissue treponemas demonstrate various antigenic properties.

Biochemical properties

The bacteria have slow metabolism, which is not ascertained in details. Genome sequence revealed treponemas to be unable to synthesize necessary growth factors (enzyme cofactors, fatty acids, nucleotides, and others). On the contrary, treponemas carry multiple transport proteins, specific to various substrates, to compensate the lack of nutrients. Carbohydrates serve as energy source in microbial metabolism owing to the presence of all glycolytic pathway enzymes in bacterial cell.

T. pallidum doesn't produce superoxide dismutase, catalase, or peroxidase.

Antigenic structure

Antigenic characteristics of *T. pallidum* are also not completely elucidated. Bacteria are considered to have many lipid and protein antigenic substances mostly with haptenic activity.

More than 100 protein antigens have been found in treponemas. Among them three core proteins of endoflagella are similar with other bacterial flagellin proteins.

Lipid antigens include phospholipid ***cardiolipin*** that shows mimicry with bovine heart lipid antigens. It is important for syphilis serological diagnosis.

Multiple lipid and protein antigens of *T. pallidum* cause hypersensitivity reactions of host immune system.

Virulence factors

T. pallidum bears membrane proteins that may function as porins and adhesins. These microorganisms are not shown to produce LPS endotoxins or clear exotoxins, but can develop cytotoxic activity against various cell cultures. Bacteria render hemolytic activity encoded by genes of five hemolytic proteins, and may produce hyaluronidase, which promotes microbial invasiveness.

Pathogenesis of syphilis is closely associated with host autoimmune reactions triggered by microbial antigens.

Resistance

Spirochetes are very sensitive to drying, heating, and action of chemical disinfectants. For instance, heating at 55°C kills them in 15 minutes. Nevertheless, they stay viable for a meaningful time in tissues especially at low temperatures. For example, bacteria survive for one day and even more in blood or plasma, stored at 4°C.

Pathogenesis and Clinical Findings in Syphilis

Syphilis is an ***anthroponotic*** disease with cyclic chronic course. It is an actual example of ***social disease***, where economic and social conditions, the state of healthcare service as well as personal lifestyle and mode of behaviour play decisive role in disease spread.

Syphilis is transmitted predominantly by ***sexual intercourse***; transmission by direct contact or via medical manipulations seems negligible.

T. pallidum penetrates through small lesions in the skin or mucosals. ***Infectious dose*** for disease is ***minimal***: as little as 1-5 microbial cells can cause the disease.

Incubation period depends on inoculated dose. A large inoculum, e.g., about 10^7 bacterial cells, results in disease appearance in 5-7 days.

There are several consequent stages in syphilis course.

After 7-90 days of incubation with average of about 3 weeks a ***hard chancre***, essential tissue lesion of ***primary syphilis***, appears. It is followed by regional lymphadenopathy.

Chancre evolves at the primary site of microbial entry. In men it usually affects penis. Anorectal chancres emerge in homosexual men. In women it predominantly occurs on vulva. Hard chancre is a painless ulcer about 0.5-3 cm with sharp margins, clean base, induration, and sometimes with purulent discharge.

In most cases chancre heals spontaneously within about 6 weeks. Nevertheless, in several weeks the disease comes into stage of "***secondary syphilis***", which results from lymphogenous and hematogenous microbial dissemination.

Secondary syphilis is characterized by skin rashes, headache, fever, malaise, lymphadenopathy, mucosal lesions, and CNS disorders. It lasts from 2-3 months to more than 1 year.

Primary and secondary syphilitic lesions contain great amount of spirochetes, being highly infectious.

In secondary stage specific immune reactions against spirochetes arise (***seropositive syphilis***). Primary syphilis is regarded as ***seronegative***, but the end of primary syphilis might be seropositive as well.

Meanwhile, hypersensitivity response doesn't provide complete microbial elimination without antimicrobial treatment; and after ***latent period*** of various duration (about 1 year and more) ***tertiary syphilis*** develops.

Tertiary syphilis affects various body's organs and tissues, especially cardiovascular system and CNS. Syphilitic aortitis damages the ascending aorta. It may happen between 10 and 30 years after primary infection.

Specific slow indurative injuries (***gummas***) emerge in central nervous system and parenchimatous organs. They are followed by necrosis and connective tissue proliferation.

The latest period of disease is characterized by profound CNS disorders (***neurosyphilis***). This period is usually regarded as seronegative because spirochetes are absent in bloodstream and antibody titers are low. The disease results in meningovascular syphilis, pareses, and ***tabes dorsalis***. Tabes dorsalis ensues from the severe injury of dorsal roots and columns of spinal cord.

Congenital syphilis results from vertical disease transmission from mother to fetus with a rate of 70 to 100% for primary syphilis. Congenital syphilis influences pregnancy outcome, thus it is often followed by spontaneous abortion, or perinatal death.

The infected infants may be asymptomatic or show various early and late manifestations, such as lymphadenopathy, hepatomegaly; skeleton and teeth lesions, CNS disorders like deafness (*Hutchinson's triad*), asymptomatic neurosyphilis, etc.

The ***immunity*** in syphilis is ***not sterile***. It always causes patient's hypersensitivity. Immune response usually doesn't prevent disease progression, but autoimmune reactions accelerate tissue damage aggravating the disease course.

Laboratory Diagnosis of Syphilis

Laboratory diagnosis rests on ***microscopical examination*** of lesion specimens for treponemas, and/or ***serological tests*** for specific antibodies.

Microscopy is the main diagnostic method for ***primary syphilis diagnosis***. It also may be used in secondary syphilis.

Specimens are collected from chancre discharge, rash elements, lymph node aspirates, etc.

Romanowsky-Giemsa stain, silver impregnation, dark field microscopy and *direct fluorescent-antibody testing* for *T. pallidum* (*DFA-TP*) are used.

Serological testing is the cornerstone for laboratory diagnosis for latent, secondary, and tertiary syphilis.

The methods of analysis include ***nontreponemal*** and ***treponemal*** reactions.

Nontreponemal reactions are employed for mass screening, whereas treponemal tests are confirmatory.

Widespread ***nontreponemal tests*** comprise two similar reactions – *Venereal Disease Research Laboratory test* (***VDRL test***) and *Rapid Plasma Reagin test* (***RPR test***). Both tests are based on flocculation reaction. In these methods a complex antigen containing lecithin, cholesterol, and purified cardiolipin is used to reveal host antibodies against cardiolipin that arise in syphilis.

The method is cheap and rapid but of limited sensitivity; and it can give false-positive data in autoimmune diseases, patients with malignancies, tuberculosis, leprosy, viral and parasitic infections, pregnancy, etc.

Wasserman reaction devised from complement fixation test can use both nontreponemal cardiolipin antigen and specific treponemal antigens for detection of anti-treponemal antibodies.

Treponemal tests comprise ***serum fluorescent treponemal antibody absorption test***, as well as *T. pallidum* ***immobilization test***, ***microhemagglutination test*** and ***ELISA*** for detection of antibodies against *T. pallidum*.

These tests use pathogenic killed or live *T. pallidum* cultures or the filtrate of virulent tissue treponemas as an antigenic source.

ELISA test is regarded as the most convenient and universal for routine laboratory diagnosis of syphilis.

PCR for *T. pallidum* DNA is swift, sensitive and reproducible method; it becomes available now in wide clinical practice.

Clinical diagnosis of ***primary syphilis*** is confirmed by positive results of microscopy and/or positive results of one nontreponemal and one treponemal test.

Secondary syphilis is diagnosed by positive data from one nontreponemal and one treponemal test.

Tertiary syphilis should be confirmed by two or more treponemal tests.

Congenital syphilis is diagnosed by clinical, serological, and direct microscopic methods. Detection of immunoglobulin M antibodies by ***fluorescent treponemal antibody absorption test*** or ***ELISA*** confirms the diagnosis.

Treatment and prophylaxis of syphilis

As *T. pallidum* has no genetic resistance to beta-lactam drugs, ***benzylpenicillin*** and its long-acting derivatives (e.g., benzathine penicillin) remain the drugs of choice for syphilis ***treatment***. Tetracyclines and macrolides can be used in case of patient's allergy to beta-lactam antibiotics.

Prophylaxis of syphilis is ***non-specific***. It requires public education, screening for syphilis, timely recognition of syphilitic cases, their adequate treatment, and improvement of socioeconomic conditions.

NEISSERIA GONORRHOEAE

The History of Discovery

Gonococci, the causative agents of gonorrhoea, were described first by A. Neisser in 1879. Later in 1885 E. Baum obtained the pure culture of these bacteria.

Classification

Gonococci belong to the family *Neisseriaceae*, genus *Neisseria*, and species *Neisseria gonorrhoeae*.

Structure and Properties of *Neisseria gonorrhoeae*

Morphology

Gonococci are similar with meningococci (about 70% of genetic similarity). Bacteria are visualized as ***gram-negative***, *bean-shaped* ***diplococci.***

Gonococci are non-sporeforming non-motile microorganisms. Unlike meningococci, *Neisseria gonorrhoeae* is lack of capsule.

The bacteria express multiple pili and fimbriae. They carry a large number of plasmids. Some of them confer resistance of gonococci to antimicrobial drugs resulting from beta-lactamase expression.

Cultivation

N. gonorrhoeae are even more fastidious than meningococci and can't multiply on basic nutrient media.

They are cultivated on media, containing blood, serum or ascitic fluid (blood, serum or ascitic agar) better in atmosphere with 5-10% CO_2 at pH 7.2-7.6. Optimal growth temperature is 37°C; the bacteria lose viability out of range 25-42°C.

Gonococci produce very small convex colonies, opaque or transparent, depending on Opa protein expression.

Biochemical properties

Gonococci are mostly aerobic or facultatively anaerobic bacteria. The bacteria yield minimal biochemical activity. They ferment solely glucose with acid end products being lack of proteolytic activity.

Similar to other members of the genus, gonococci produce oxidase and catalase.

Antigenic structure

N. gonorrhoeae harbors various antigenic determinants of polysaccharide and protein nature. The pathogens are able to alter surface antigen expression to evade host immune response. Bacterial pili contain protein ***pilin***, which significantly varies among gonococcal strains (about 100 serovars). Gonococci express a number of ***porins***, namely PorA and PorB proteins. Multiple serovars are determined according to Por antigen variations.

Adhesive ***Opa*** (***opacity***) proteins also render antigenic activity.

Polysaccharide epitopes of gonococci are confined within bacterial cell wall ***lipooligosaccharide*** (***LOS***).

N. gonorrhoeae can switch the synthesis of various antigenic molecules, e.g pilins, Opa proteins or LOS residues triggering alternate gene expression. Overall, gonococci are regarded as the bacteria with ***highest genetic variability*** and genetic exchange with other bacterial species.

Virulence factors

Bacterial adhesins, including pili and Opa proteins promote microbial attachment to the host cells. ***Opa proteins*** principally bind to the cells bearing ***CD66*** *carcinoembryonic antigen*.

Opa and ***Por*** proteins stimulate intracellular invasion of gonococci and ***inhibit phagocytosis***, preventing phagosome-lysosome fusion.

Microbial lipooligosaccharide displays evident ***endotoxin*** activity. LOS antigenic mimicry with human glycosphingolipids support gonococci to escape host defensive reactions.

Gonococci produce ***IgA1 protease*** that cleaves human mucosal IgA1. Many bacterial strains express plasmid-encoded ***beta-lactamases***.

Resistance

Gonococci demonstrate a low viability, being very sensitive to external influences. They can't resist cooling, drying, or UV irradiation. Gonococci best survive in the moist conditions in various human discharges.

Bacteria are killed at temperature of 56°C within 5 minutes. They are readily inactivated by treatment with ordinary disinfectants.

Pathogenesis and Clinical Findings in Gonorrhoea

Gonococcus is the strictly ***human*** pathogen.

Gonorrhoea is a typical ***sexually transmitted disease*** that affects predominantly urogenital tract. Unprotected sexual intercourse results in 50% likelihood of disease contraction in women and 30-50% in men.

Also gonococci produce gonorrhoeal conjunctivitis in adults and ***ophthalmia neonatorum*** (or ***blennorrhoea***) in newborn infants transmitted by ***contact*** route.

Infectious dose of bacteria is generally ***low*** – about 10^3 cells of virulent strains

Only piliated opaque gonococcal cultures, containing multiple adhesins (e.g., Opa proteins), are able to adhere and invade host tissues.

Gonococci attach to mucosa of urogenital tract, rectum, or eye, and induce acute inflammation. They stay viable within phagocytes impairing their bactericidal activity (*incomplete phagocytosis*).

In males they cause specific urethritis with suppurative discharge followed by dysuria with frequent painful urination. The process often involves epididymis. When untreated, the disease produces chronic inflammation with extensive tissue fibrosis and seminal duct obliteration that may cause male ***infertility***.

In females the primary penetration of bacteria occurs in the endocervical epithelium. The infection extends to the urethra and vagina, and affects uterine tubes thus provoking salpingitis. Fibrosis and obturation of uterine tubes result in female ***infertility***. Female gonorrhoea may be asymptomatic.

If not treated the disease easily becomes ***chronic***.

In some rare cases the infection breaks tissue barriers, and the bacteria enter the bloodstream. This leads to hematogenous microbial spread with hemorrhagic skin rashes. Gonococcal dissemination may produce specific arthritis or endocarditis.

Blennorrhoea or ***ophthalmia neonatorum*** evolves as the result of neonate infection, when newborns pass through infected maternal canal. Specific gonococcal eye injury can cause infant blindness.

The ***immunity*** doesn't confer the resistance against gonococci albeit specific antibodies and immune cells can appear in human secretions.

Laboratory Diagnosis of Gonorrhoea

Specimens are collected from the discharge of urethra, vagina, vulva, cervix, rectum or conjunctiva in case of *ophthalmia neonatorum*.

Gram-stained smears of secretions show typical gram-negative bean-shaped cocci within polymorphonuclear leukocytes (incomplete phagocytosis) or extracellularly.

More sensitive and specific is ***immunofluorescent*** test.

Gonococcal antigens in clinical specimens are determined by ELISA.

To obtain ***microbial culture*** the collected specimens are inoculated immediately into serum or ascitic agar. The media are supplemented with antibiotics, suppressing concomitant bacteria and fungi (vancomycin, amphotericin or ristomycin). For men the culture is not necessary in case of positive microscopic examination, but cultures for women are indispensable.

After incubation for 48 h in chamber with 5% CO_2 the specimens can yield pure cultures. They are further confirmed by microscopy with Gram stain, fermentation tests and microbial antigens determination.

Serological reactions are of limited use in gonorrhoea.

As confirmatory tests for detection of microbial nucleic acids, ***PCR*** and other *nucleic acid amplification tests* (***NAATs***) are used.

Treatment and Prophylaxis of Gonorrhoea

Because of rapidly growing resistance of gonococci to antimicrobial agents, third generation cephalosporins (e.g., ceftriaxone) and macrolides/azalides (azithromycin) are currently recommended for ***treatment*** of gonorrhoea..

However, in 2011 first ceftriaxone-resistant isolates of gonococci were registered. Later in 2013 a new drug combination of azithromycin and gentamycin was introduced into clinical practice that is efficient against multiresistant gonococcal strains.

For treatment of chronic gonorrhoea the injections of gonococcal killed vaccine can be administered to stimulate host immunity.

For ***protection of newborns*** against ophthalmia neonatorum, urgent eye instillations of ***sulfacetamide*** (sulfacyl-sodium) solution as well as applications of tetracycline or azithromycin ophthalmic ointments are administered immediately after birth.

PATHOGENIC UROGENITAL CHLAMYDIAE

The History of Discovery

Primary discovery of chlamydial inclusion bodies in conjunctival exudate of patient with trachoma was made in 1907 by S. Prowazek and L. Halberstдdter. They found microbial microcolonies later termed as *Halberstдdter-Prowazek bodies* enwrapped within common coat in the cytoplasm of infected cells. Hence, these and other similar bacteria were termed "*Chlamydia*" (from Gr. *chlamyda* that means "cloak").

Classification of Chlamydiae

The order *Chlamydiales* includes the family *Chlamydiaceae*; pathogenic represenatives pertain to genera *Chlamydia* and *Chlamydophila*.

Human pathogen *Chlamydia trachomatis* causes ***trachoma***, ***lymphogranuloma venereum*** or Nicolas-Favre disease, ***inclusion conjunctivitis***, and numerous ***nongonococcal urogenital infections*** like urethritis and salpingitis.

The genus *Chlamydophila* comprises two species pathogenic for humans – *C. pneumoniae* and *C. psittaci*.

C. pneumoniae causes human pneumonia, bronchitis and sinusitis, whereas *C. psittaci* is the causative agent of avian disease ornithosis (or psittacosis) that in some cases may occur as severe respiratory infection in humans.

Structure and Properties of Chlamydiae

Morphology and life cycle

Chlamidiae are the ***obligate intracellular parasites***. They are non-motile and non-sporeforming.

Bacteria are of very small sizes and have two stages in life cycle – *elementary bodies* and *reticulate bodies.*

Elementary bodies measuring 0.2-0.3 μm possess infectious properties being capable of ***invading*** the host cells.

In the infected cells elementary bodies transform into ***vegetative reticulate*** inclusions 0.8-1.5 μm in size. They might be covered with capsule. After several reproductions reticulate bodies convert again into elementary invasive forms.

The whole developmental cycle takes about 48-72 hours.

According to their structure, chlamydiae are gram-negative bacteria with atypical peptidoglycan without acetylmuramic acid but with multiple cystein-containing peptide cross-bridges.

Chlamydiae are primarily visualized by ***Romanowsky-Giemsa*** stain (reticulate bodies produce blue inclusions attached to cell nuclear membrane, while elementary bodies stain purple). Intracellular detection of bacteria is also performed by ***immunofluorescence technique***.

Cultivation

As the obligate intracellular parasites, chlamydiae grow in cultures of a variety of eukaryotic cell lines.

McCoy cells are commonly used to isolate these pathogens. All types of chlamydiae proliferate in embryonated eggs, particularly in the yolk sac. Various animal models are used also for cultivation, e.g. mice.

Biochemical properties

In general, chlamidiae render weak biochemical activity. Bacteria are unable to synthesize ATP and need the host cell for energy and nutrient donations.

Some chlamydiae demonstrate endogenous metabolism like other bacterial representatives. They can liberate CO_2 from glucose, pyruvate, and glutamate; they also contain dehydrogenases.

Antigenic structure

Chlamydiae possess group-specific antigens. These are heat-stable lipopolysaccharides.

Serovar-specific antigens are mainly ***outer membrane proteins*** (***OMP***). *Major outer membrane protein* (***MOMP***) covers about 60% of total amount of proteins in chlamydial cells. Other protein antigens of microbial outer membrane are variable (Pmp, OmcA, OmcB and others).

Antigenic proteins are also found in the coat encasing bacterial intracellular inclusions (Inc proteins).

Specific antigens are shared by only a limited number of chlamydiae. Fifteen serovars of *C. trachomatis* have been identified (e.g., A, B, Ba, C; D-K; L1-L3).

Virulence factors

Virulence factors of chlamydiae are not completely elucidated.

Microbial LPS displays proinflammatory properties as *endotoxin*.

The proteins of outer membrane such as ***MOMP*** are the bacterial ***adhesins***. Together with cystein-containing chlamydial proteins they suppress phagocytosis inhibiting phagosome-lysosome fusion.

Heat shock proteins ***hsp60*** and others stimulate cellular inflammation.

Chlamydiae possess ***type III secretion system (T3SS)*** with activity of injectisome. The structures of T3SS are responsible for microbial invasiveness and intracellular persistence.

For instance, ***effector protein*** *TARP* after injection into the cell stimulates cytoskeleton remodelling and next membrane folding. It leads to engulfment of attached bacteria and their entry into the epithelial cells.

Another ***effector protein*** *CPAF* with proteolytic activity destroys intracellular regulatory proteins, thus preventing the apoptosis of infected cells and presentation of chlamydial antigens.

Resistance

In general, chlamydiae demonstrate a low environmental resistance. More stable are elementary bodies, which stay viable for 5-10 min within droplet aerosol phase. The temperatures above 40^{o}C and pH fluctuations rapidly inactivate bacteria. Nevertheless, their survival might be longer at low temperatures and in clinical samples with high protein contents.

Chlamydiae are sensitive to all conventional antiseptics and disinfectants.

Pathogenesis and Clinical Findings in Chlamydial Urogenital infections

Humans are the natural hosts for *C. trachomatis*. The bacterium causes various human infections depending on the microbial serovar.

Serovars A, Ba, B and C are the agents of *trachoma*; serovars from D to K are responsible for *urogenital infections*, and L-1, L-2, L-3 serovars cause *lymphogranuloma venereum*.

Trachoma is an ancient eye disease. It is a chronic keratoconjunctivitis that begins with acute inflammatory changes in the conjunctiva and cornea and progresses to scarring and blindness.

Also *C. trachomatis* cause numerous ***urogenital infections***. Bacteria of ***serovars D-K*** cause sexually transmitted diseases and may also produce the specific infection of the eyes (***inclusion conjunctivitis***).

The bacteria bind to epithelial cells by multiple adhesins, enter the infected cell by the action of type III secretion system and impair normal cellullar metabolism. Chlamydia persistence stimulates chronic inflammatory reactions within urogenital tract that may lead to sclerosis. Propagation of chlamydiae followed by the egress of elementary bodies by lysis or membrane body extrusion results in degradation of urogenital epithelium.

In males *C. trachomatis* provokes ***nongonococcal urethritis*** and epididymitis. In females *C. trachomatis* causes urethritis, cervicitis, and

pelvic inflammatory disease, which can lead to sterility and predisposes to ectopic pregnancy.

Up to 50% of nongonococcal urethritis in men or the urethral dysuria in women is associated with chlamydiae. Overall, *C. trachomatis* annually causes more than 140 mln cases of sexually transmitted infections worldwide.

The infection may stay long asymptomatic but transmissible to other persons.

The newborns acquire the chlamydial infection, when passing through the infected maternal birth canal. From 20 to 50% of newborns may acquire the infection, 15-20% of them display eye symptoms and 10-20% demonstrate the involvement of respiratory tract.

Inclusion conjunctivitis of the newborns commences as suppurative conjunctivitis arisen in 1-2 weeks after the delivery. It is manifested like chronic chlamydial infection similar to childhood trachoma.

Laboratory Diagnosis of Chlamydial Urogenital Infections

A cytology brush or swab is used to detach epithelial cells 1-2 cm deep from the endocervix. A similar method is applied to collect ***specimens*** from the vagina, urethra, or conjunctiva. Biopsy specimens of the uterine tube or epididymis can also be examined.

The presence of chlamydia inclusions in smears is determined by microscopy with ***Romanowsky-Giemsa*** stain and ***immunofluorescence*** microscopy.

The swab specimens should be placed into chlamydia transportation medium and kept at refrigerator temperature before transportation to the laboratory.

McCoy cells grown in monolayers are inoculated for ***culture***. The inoculum from the swab specimen is incubated at 37°C for 48-72 hours. The monolayers are examined by ***direct immunofluorescence*** to visualize the cytoplasmic inclusions. This method of chlamydial cultures demonstrates about 80% sensitivity and near 100% specificity.

Nevertheless, cultural tests remain laborious and cumbersome. Therefore, the laboratory diagnosis of chlamydial infections in clinical practice is mainly based on ***PCR*** as the highly sensitive, specific and reproducible ***molecular genetic test***. It is more sensitive than culture and other nonamplification tests. The specificity of PCR appears to be close to 100%.

Direct fluorescent antibody assay and enzyme-linked immunoassay (ELISA) are used to detect *C. trachomatis* by their antigens.

Serological diagnosis of chlamydial infections (e.g., by ELISA) indicates the growth of serum antibodies against the pathogen. The rise of levels of specific antibodies occurs during and after the acute chlamydial infection.

Treatment of Urogenital Chlamydioses

Macrolides and azalides (e.g., azithromycin) are commonly used for ***treatment*** of urogenital chlamydial infections. Erythromycin is given to pregnant women. Tetracyclines (e.g., doxycycline) can be administered as well.

Aminoglycosides and β-lactams are clinically inefficient due to the poor availability of chlamydiae inside the cells. Topical tetracyclines or macrolides are administered in case for inclusion conjunctivitis, sometimes in combination with a systemic drug.

Efficient vaccines for prevention of chlamydial infections are not yet elaborated.

PATHOGENIC UROGENITAL MYCOPLASMAS

Classification of Uropathogenic Mycoplasmas

As it was mentioned above in Chapter 6, mycoplasmas pertain to the separate class *Mollicutes*, order *Mycoplasmatales*, and family *Mycoplasmataceae*, which includes two genera with pathogenic representatives: *Mycoplasma* and *Ureaplasma*.

In certain clinical conditions *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, and *Mycoplasma genitalium* can cause human ***nongonococcal urethritis*** and some other urogenital disorders. These ailments are usually not found as monoinfection but predominantly as the tight association of various urogenital pathogens.

The role of other mycoplasmal species in pathology of urogenital tract remains elusive.

Pathogenic Mycoplasmas – Basic Characteristics

The structure and common properties of mycoplasmas were already described earlier (see Chapter 6 for details).

Briefly, mycoplasmas are the smallest pleomorphic bacteria, which are ***lack of the cell wall***. Their cells are covered with thick lipid-containing membrane. They are facultative anaerobes or microaerophils with reduced metabolism.

Mycoplasmas are ***membrane parasites***, but they can grow on special nutrient media supplemented with serum, ascitic fluid, lipoproteins and sterols. After growth the bacteria yield round colonies of minimal sizes looking like "*fried eggs*".

When cultured within the cell lines and chicken embryos, mycoplasmas become closely attached to the membranes of affected cells.

Basic antigens of mycoplasmas are glycolipids and proteins of variable structure.

The virulence factors of mycoplasmas are not well-defined. The bacteria can generate cytotoxic *hydrogen peroxide* and *superoxide radicals*. Some strains may express hemolysins. Membrane fractions of mycoplasma play the role of *superantigens*. All mycoplasmas possess multiple adhesins.

The bacteria are very sensitive to external influences. They are easily inactivated under the action of conventional antiseptics and disinfectants.

Pathogenesis and Clinical Findings in Mycoplasmal Urogenital Infections

Various mycoplasmas have non-equal association with urogenital disorders. Moreover, such a relationship is hard to establish due to relatively low virulence of these bacteria. Isolated mycoplasmal culture may occur as nonpathogenic concomitant bacteria, which only follow infection process. In addition, these microbials frequently play a role in urogenital disorders only in closest associations with other urogenital pathogens (*trichomonads*, *gardnerellas*, *chlamydiae* and many others). Thus it remains difficult to evaluate real contribution of mycoplasmal infection into urogenital pathology.

Mycoplasma hominis was demonstrated in some patients with pyelonephritis, urethritis, prostatitis, salpingitis and tubo-ovarian abscesses. Systemic mycoplasmal infection may provoke postabortal or

postpartum fever in females. *Mycoplasma genitalium* can be revealed in patients with nongonococcal urethritis. *Ureaplasma urealyticum* can be often found in female urogenital tract, but its role in women genital disorders remains unclear. Ureaplasmas may be isolated also in some cases of nongonococcal urethritis in men.

Laboratory Diagnosis of Mycoplasmal Urogenital Infections

Urethral and vaginal scrapes or swabs, genital secretions are largely used for ***specimen*** collection.

PCR has become the most valuable, rapid and sensitive test for mycoplasma detection. The only drawback of the method depends on reaction inability to discriminate viable and degraded microbial cells by their nucleic acids. The latter condition may rarely cause false-positive test results.

For culture the specimens are inoculated into special solid or liquid media and incubated for 1-2 weeks. After incubation the growth of minute characteristic "fried egg"-like colonies is observed. Further subculture is useful to identify bacteria by immunofluorescence test. Mycoplasma culturing is usually available in specialized reference laboratories.

Serological tests are of limited significance in local mycoplasmal infections.

Prophylaxis and Treatment

The bacteria are resistant to antibiotics that inhibit cell wall synthesis (e.g., beta-lactams and vancomycin). Macrolides, azalides and tetracyclines (doxycycline) are preferable in ***treatment*** of urogenital mycoplasmal infections. Some strains develop increased resistance to tetracyclines and macrolides.

Specific prophylaxis of infection is not available. Non-specific measures are used to prevent disease transmission.

Chapter 9

CAUSATIVE AGENTS OF BACTERIAL ZOONOZES: PLAGUE, ANTHRAX, BRUCELLOSIS, AND TULAREMIA

YERSINIA PESTIS – CAUSATIVE AGENT OF PLAGUE

The History of Discovery

Plague is a highly devastating epidemic disease still known from the times of antiquity. It followed human civilization from the deep past.

Three global pandemics of plague were registered in the written history – the Justinian Plague of 541 AD that affected the all ancient world, lasted for two centuries and caused about 25 mln deaths; the Great Plague or "Black Death" of Middle Ages that killed 60% of European population, and the last plague pandemic started in China in the 1860s and continued for several decades with about 10 mln victims.

In XX century the total number of plague cases gradually declined. Nowadays between 1,000 and 2,000 plague reports are delivered annually to WHO, but this statistics is regarded as seriously underestimated.

The causative agent of ***plague***, *Yersinia pestis*, was discovered by the French microbiologist A. Yersin in Hong Kong in 1894 during the last global pandemic of the disease.

Classification of *Yersinia pestis*

Yersinia genus belongs to the family *Enterobacteriaceae*.

Yersinia pestis, a causative agent of plague is the most virulent yersinia representative.

Structure and Properties of *Yersinia pestis*

Morphology

All yersiniae are similar with other enterobacteria – small polymorphic gram-negative rods that have characteristic bipolar stain. Stained microbial cells look like "closed safety pins" on microscopy.

Y. pestis are nonmotile enterobacteria, in contrast to other yersiniae, which express flagella.

In smears from tissues and cultures *Y. pestis* is found to have a delicate capsule.

Cultivation

Yersiniae can grow on ordinary nutrient media. The optimal temperature for cultivation is 25-30°C.

The growth on blood agar after 48 h of incubation at 35°C yields gray-white opaque colonies 1-2 mm in diameter usually without hemolysis. The colonies resemble "fried-egg" or "crumpled lace handkerchief" at appearance.

Various lactose-containing media (McConkey, or EMB agar) and several special media are used for yersiniae cultivation. Small lactose-negative colonies arise after 24 h of incubation at 35°C.

In meat broth the cultures form a pellicle on the surface with threadlike growth resembling stalactites.

Y. pestis is virulent in ***R form***.

Biochemical properties

Yersiniae are facultatively anaerobic bacteria. As all enterobacteria, they are catalase positive and oxidase negative.

Y. pestis has rather weak and variable biochemical activity. The bacterium ferments glucose, maltose, galactose, mannitol and some other carbohydrates with acid end products, but can't metabolize sucrose, and in most cases lactose. It reduces nitrates to nitrites.

Y. pestis neither liquefy gelatin, nor produce indole. They are urease negative.

Antigenic structure

Y. pestis express many antigens and toxins that act as virulence factors. ***O-antigen*** contains lipopolysaccharides that have ***endotoxic*** activity, when released. Capsular ***K-*** (or ***F1***) antigen of glycoprotein nature is shown to protect bacteria against phagocytosis.

Protein V-Ag and lipoprotein W-Ag can also develop anti-phagocytic activity.

Virulence factors

All virulence factors of *Y. pestis* are under the tight genetic control.

A 72-kb ***virulence plasmid*** (***pYV*** – plasmid of *Yersinia* virulence) is responsible for microbial adherence, invasiveness, intercellular spread, and ability to survive and propagate within host lymphoid tissues. It is essential for mirobial virulence; avirulent strains lack this plasmid. The plasmid contains yersinia pathogenicity island or ***Yop virulon***.

Yop virulon encodes invasive *yersinia outer proteins* (***Yop proteins***) and structures of type III bacterial secretion system (***injectisome***, or ***needle complex***), composed of about 30 protein units (so-called ***Ysc proteins***). Needle complex promotes microbial adherence to epithelial or immune cells and delivers invasive Yop proteins into cytoplasm of the host cells. Injected Yops impair the dynamics of the cytoskeleton, allow microbial penetration and intracellular spread, thereby promoting further bacterial invasion.

Yop proteins ***sharply diminish*** the production of proinflammatory cytokines by macrophages and other immune cells, thus maintaining bacterial survival within immune cells and tissues. Also they stimulate ***macrophage apoptosis***.

Among secreted ***exotoxins***, one is lethal for mice in amounts of 1 ng. This deleterious protein substance is extremely cardiotoxic for experimental animals. Its role in human infection is not clearly elucidated.

K- (or ***F1***), ***V-*** and ***W-antigens*** protect bacteria against phagocytosis.

Y. pestis expresses ***plasminogen activator*** – a potent agressive enzyme complex. At 28°C (the normal temperature of the flea body) it shows ***coagulase*** activity supporting bacterial dwelling within insect vector, whereas at 35-37°C (human body temperature) it affords ***fibrinolysin*** activity, thereby breaking down the tissue matrix and promoting microbial invasion. The latter is additionally stimulated by microbial ***hyaluronidase***.

Also the bacteria produce bacteriocins (or ***pesticins***) that antagonize normal microbiota.

Bacterial ***lipopolysaccharide*** has potent ***endotoxic*** activity. In human body conditions at 37^{o}C the structure of lipid part of endotoxin alters, and modified LPS loses its capacity to stimulate macrophages thus maintaining microbial survival.

Resistance

The plague agents can withstand low temperatures. At 0°C they live for 6 months. *Y. pestis* survives in water for 30 days; in milk for 90 days; in bubonic pus for 20-30 days; in sputum for 10 days.

Y. pestis is very sensitive to drying and high temperatures. Boiling kills the microbials within 1 minute, when heated to 60°C they are inactivated in 1 hour. Standard disinfectants in ordinary concentrations (e.g., 5% phenol) readily destroy them in 5-10 minutes.

Epidemiology, Pathogenesis and Clinical Findings in Plague

Plague is a ***zoonotic*** disease of rodents and other animals that is usually transmitted to humans ***via fleabites***.

Rodents, among them black rats, grey rats, mice, gophers, marmots (tarbagans) and many others are susceptible to plague. More than 300 rodent species as primary ***sources of infection*** may spontaneously contract the disease.

When an insect ***vector*** (flea) feeds on a rodent infected with *Y. pestis,* the ingested bacteria multiply in the gut of the flea. Microbial cells block vector's digestive tract owing to coagulase action. Then the flea attacks and bites the mammal host, and flea gut contents contaminated with *Y. pestis* become expelled into the bite wound.

At the temperature of the flea body 28°C *Y. pestis* neither secrete virulence proteins, nor alter the structure of LPS and express capsular F1-Ag.

In human body at 37°C the inversion of microbial metabolism occurs. By activation of injectisome *Y. pestis* delivers virulence effector proteins into macrophages and other immune cells. They paralyze the activity of innate immune response with profound inhibition of cytokine secretion. Alteration of LPS structure and expression of bacterial F1-, V- and W-Ags protects the bacteria against fagocytosis.

Fibrinolysin activity of bacterial ***plasminogen activator*** and hyaluronidase facilitate microbial dissemination.

Thus, the powerful mechanisms of ***suppression of innate immunity*** essential for *Y. pestis* create the conditions for generalized ***systemic*** devastating infection.

Depending on the location of the pathogen, virulence of the microbe, and host immunity human plague is manifested in three major forms: ***bubonic***, ***pneumonic***, and ***septicemic***. More seldom are cutaneous and intestinal forms.

Fleabite results in the ***bubonic form*** of plague, characterized by the sudden rise of fever and an extremely painful ***lymphadenitis*** known as ***bubo***. It usually appears in the groin or axillae.

Skin dark-purple lesions may develop during the systemic stage of the infection. They rapidly become necrotic and likely account for the plague name "*black death*".

The term ***septicemic plague*** describes fulminant disseminative infection without bubonic lymphadenitis.

Pneumonic plague arises after hematogenous spread of yersiniae from a bubo to the lungs (secondary pneumonic) or via the direct inhalation of pathogens.

Direct inhalation of *Y. pestis* sharply accelerates the disease transmission between humans (***primary pneumonic*** plague).

The pneumonic disease is highly contagious and easily spread among individuals via ***airborne route***.

As the result of systemic infection, hemorrhagic and necrotic lesions emerge in all organs and tissues. They lead to meningitis, pneumonia and other inflammatory disorders followed by kidney, liver and cardiovascular failure. Disseminated intravascular coagulation (***DIC***) entails hypotension and collapse.

Administration of antimicrobial agents (e.g., gentamycin or tetracycline) early in the course of the disease can reduce mortality from approximately 50% in untreated plague cases to about 5-10%.

After patient's recovery a ***stable immunity*** of long duration is acquired.

Due to its prominent virulence, rapid air-droplet spread of pneumonic disease and high fatality of infection if not treated *Y. pestis* is ascertained as the potential agent of bioterrorism and biological warfare.

According to United States regulations, the list of "Biological Select Agents or Toxins" comprises microorganisms and their toxins that possess "...the potential to pose a severe threat to public health and safety".

Y. pestis stays in Tier 1 of US Select Agents list (the highest rank of public threat). Criteria for Tier 1 are summarized as follows: "(1) ability to produce a mass casualty event or devastating effects to the economy; (2) communicability; (3) low infectious dose; (4) history of or current interest in weaponization based on threat reporting".

Laboratory Diagnosis of Plague

For isolation of *Y. pestis* the ***specimens*** from blood, sputum, or lymph node aspirates are taken. The bacteria are also recovered from autopsy material (organs, blood, lungs, lymph node samples), rodent corpses, fleas, foodstuffs, water, etc.

At the first stage of examination *Y. pestis* are often detected by ***microscopy*** in smears stained by Gram method or methylene blue.

Immunofluorescence test is used for rapid detection of bacteria in clinical samples.

Microbial nucleic acids are detected by ***PCR***.

Cultures of *Y. pestis* should be operated in special biosafety facilities (***BSL-2*** – biosafety level 2) with minimization of procedures that may create aerosols.

Microbial ***isolation*** is performed in ordinary media supplemented with antiseptics, e.g., gentian violet that inhibits concomitant microflora. Growth on media incubated at 35-37°C is slower than growth at 28°C or room temperature.

Any suspected *Y. pestis* isolate should be delivered to the state reference laboratory for identification.

For ***biological tests*** (animal experimental infection) isolated pure cultures or specimens are inoculated into guinea pigs. If plague agents are present, the animals die in 5-7 days.

Y. pestis is further identified according to their biochemical and antigenic properties and by phagotyping. It should be differentiated from other yersiniae, e.g. the causative agent of pseudotuberculosis.

Serological testing of patients detects arisen antibody levels to *Y. pestis* by agglutination or ELISA test.

Treatment and Prophylaxis of Plague

Aminoglycoside antibiotics (streptomycin or gentamycin) are used for the ***treatment*** of the plague. The drugs are effective even in pneumonic plague. Good results were obtained from a combination of streptomycin with tetracyclines and passive treatment with anti-plague immune globulin.

Specific ***prophylaxis*** is afforded with live ***EV vaccine***. The persons from the groups of risk working in the areas of infection are vaccinated (e.g., medical personnel, veterinary workers, hunters, herdsmen, etc.) Immunity lasts for about 1 year. Booster injections are possible in 6-12 months. The efficacy of vaccination is generally moderate.

B. ANTHRACIS – CAUSATIVE AGENT OF ANTHRAX

The History of Discovery

The bacterial origin of ***anthrax*** was primarily noted by A. Pollander (Germany) in 1849, by K. Davaine (France) in 1850, and by F. Brauell (Russia) in 1854.

First isolation of anthrax agent was fulfilled by R. Koch in 1876.

In Russia the disease was named as Siberian sore owing to the large epidemic of 1786-1788 in the Urals, described by S. Andreyevsky.

In Germany the infection is known as spleen fever.

Classification of Bacillae

Bacillus genus pertains to the same name family *Bacillaceae*.

Most members of the genus are saprophytic organisms prevalent in soil, water, air, and on vegetations, such as *Bacillus cereus* and *Bacillus subtilis*. These bacteria may occasionally produce disease in immunocompromised persons (e.g., meningitis, endocarditis, acute gastroenteritis, etc.)

B. anthracis, which causes anthrax, is the principal pathogen of the genus.

Structure and properties of *B. anthracis*

Morphology

B. anthracis are viewed as the large gram-positive rods (1-1.5 μm in breadth and 4-10 μm in length). The microbials are nonmotile, encapsulated, and arranged in chains (***streptobacilli***).

In stained smears the ends of the bacilli appear to be sharply cut across, resembling *bamboo canes*. Peptide ***capsule*** is usually evident in samples from infected tissues.

The bacilli produce oval subterminal ***spores*** that not exceed the width of bacterial cell. Spores are formed only in the presence of oxygen.

Cultivation

B. anthracis grows well in ordinary media and sheep blood agar at 37°C, usually forming nonhemolytic rough colonies (***R forms***) after overnight incubation.

The round colonies are usually flat or slightly convex with irregular edges, sometimes curly tailing edges are observed. It was historically indicated that they resemble the "*head of a medusa*" or "*lion mane*."

The smooth S forms display low virulence or are completely avirulent.

When grown on penicillin-containing meat-peptone agar, the bacilli are transformed into globules that become arranged as a necklace ("*pearl necklace*").

Broth cultures of the anthrax bacilli produce flocculent growth as "*cotton wool*" near the bottom of the tube.

Growth of *B. anthracis* in gelatin stabs with substrate liquefaction resembles an inverted "*fir tree*".

Biochemical properties

The anthrax bacilli are ***aerobic*** and facultatively anaerobic. They have potent and versatile biochemical activity.

B. anthracis expresses various enzymes – peroxidase, catalase, lipase, amylase. Bacteria utilize proteins producing ammonia and hydrogen sulfide. In addition, anthrax bacilli liquefy gelatin and cause late liquefaction of coagulated serum. They slowly reduce nitrates to nitrites and coagulate milk.

B. anthracis ferments glucose, maltose, sucrose, dextrin, etc. with acid production.

Antigenic structure

The anthrax bacilli carry ***capsular protein*** and thermoresistant ***polysaccharide cell wall antigen***.

The polysaccharide antigen remains stable for the long period of time in tissues obtained from animal carcasses. The presence of this antigen in raw materials is determined by ***Ascoli's thermoprecipitin test*** – a boiled *B. anthracis* extract containing thermoresistant Ag yields a precipitin reaction with the specific serum.

The capsular antigen is composed of poly-D-glutamic acid

B. anthracis produces also a special antigenic fraction, referred to as ***protective antigen***. This antigen is a thermolabile protein with marked immunogenic activity. It takes an active part in metabolism of microbial toxins.

Virulence factors

The virulence factors of *B. anthracis* include two ***exotoxins*** and ***antiphagocytic*** polypeptide ***capsule***. The loss of the capsule abolishes the virulence of bacteria.

The genes encoding virulence factors of *B. anthracis* are located in two separate plasmids.

The ***anthrax toxins*** are composed of three proteins: ***PA*** (*protective antigen*), and ***EF*** (*edema factor*) or ***LF*** (*lethal factor*).

Protective antigen plays a role of receptor and transport subunit for both microbial toxins. The PA molecule attachs to specific receptors on the host cell membrane. PA is further cleaved by a cellular protease, producing a PA fragment that functions as a specific receptor for edema factor (***EF***) and lethal factor (***LF***).

Edema factor is an ***adenylate cyclase***; after binding to PA it forms a toxin known as ***edema toxin***. It suppresses the activity of macrophages and increases vascular permeability resulting in tissue edema.

Lethal factor coupled with PA creates cytotoxic ***lethal toxin***, which is the major virulence factor of *B. anthracis*.

LF itself is ***zinc-containing metalloprotease*** that with high specificity destroys key intracellular regulatory enzyme, namely ***kinase*** *of mitogen-activated protein kinase* (***MAPK kinase***).

Inactivation of MAPK kinase by ***lethal toxin*** profoundly disorganizes intracellular metabolism eventually resulting in cell death.

Both lethal and edema toxins are delivered into the host cell via receptor-mediated endocytosis. Initially confined within endosome, LF and EF cross endosomal membrane and enter the cytoplasm of affected cell through the channel made by protective antigen subunit.

Resistance

The intrinsic toughness of *B. anthracis* spores is outstanding: they may survive in soil for decades (at least more than 50 years). They are grossly more resistant to disinfectants than the vegetative cells. The vegetative bacteria are killed in 15 minutes at 60°C and in 1-2 minutes at 100°C. The spores are thermostable, and withstand boiling for 15-20 minutes or autoclaving at 110°C for 5-10 minutes. They are gradually destroyed by 1% formaldehyde and 10% sodium hydroxide within 2 hours.

Epidemiology, Pathogenesis and Clinical Findings in Anthrax

Anthrax is a typical ***zoonosis***. It is enzootic in many parts of the world. The potential ***source of infection*** is the vast number of wild and domestic animals (e.g., cattle).

Herbivores become infected with anthrax by grazing in pastures that are contaminated with spores. The animal infection results in bacterial propagation that leads to environmental contamination with vegetative microbial cells. They subsequently sporulate and persist in the soil for 50 years and even more. Animal carcasses are highly infectious. Biting flies can become vectors for the spread of anthrax.

As the environmental conditions play a substantial role in preservation and spread of anthrax germs, the infection caused by *B. anthracis* is referred to as ***sapronosis***.

Contact with animals (butchering, skinning, or exposure to hides or wool), and consumption of contaminated meat are the risk factors for infection transmission to humans. The incidence of inhalation anthrax is considerably reduced by decontamination procedures for wool and hair.

Depending on the primary portal of entry, anthrax cases demonstrate highly variable clinical manifestations.

In ***cutaneous anthrax***, spores are introduced into the skin by ***direct contact***. Germination occurs within hours, and vegetative cells produce anthrax toxin. The disease usually develops within 1-7 days after entry.

Primary papular-vesicular skin lesion is next changed by ulceration with formation of blackened necrotic ***eschar*** or ***anthrax carbuncle*** (malignant pustule). This lesion is usually painless. A regional lymphadenitis is commonly observed in these patients. Eventually eschar dries, loosens, and separates; spontaneous healing occurs in 80 to 90% of untreated cases. Bacterial dissemination may lead to systemic infection with high fever and possible lethal outcome.

In case of ***inhalation anthrax*** (***wool-sorter's disease***), the spores are aerosolized and enter the alveoli of the lungs. The ***incubation period*** in inhalation anthrax may last up to 6 weeks.

The spores are ingested by alveolar macrophages and begin to germinate moving to mediastinal lymph nodes. It results in hemorrhagic mediastinitis and massive *B. anthracis* bacteremia, accompanied by secondary pneumonia. Meningitis may also occur.

The disease manifests by fever, tachypnea, and hypoxia accompanied by hypotension. Severe ***respiratory distress syndrome*** leads to lethal outcome within 24 h of the primary phase of infection.

There is *no individual human-to-human transmission* of ***inhalation anthrax***; nevertheless, this form of disease might be contracted as the result of potential bioterrorist attack due to the high stability of microbial spores.

Accidental ***gastrointestinal anthrax*** arises under the ingestion of contaminated meat that was not thoroughly cooked. The course of the disease is severe, fatality is high.

B. anthracis bacteremia occurs in all three forms of human anthrax and it is observed in literally all death cases. Cutaneous anthrax is the most frequent form of disease (95%), next is inhalation anthrax (5%). Gastrointestinal anthrax is extremely rare and may be seen in less than 1% of all clinical cases.

Due to its evident threat to personal and public health, *B. anthracis* is also placed into Tier 1 of US list of "Biological Select Agents or Toxins" comprising the most dangerous microbial pathogens. *Bacillus anthracis* ranks high in the list of potential agents of bioterrorist attacks.

Laboratory Diagnosis of Anthrax

Depending on the type of infection, *B. anthracis* may be isolated from various ***samples***: cutaneous lesions, respiratory specimens, stool or other gastrointestinal excretions, blood or cerebrospinal fluid.

The ***microscopy*** of Gram-stained smears reveals the presence of characteristic capsulated bacilli, arranged in chains that permits a preliminary diagnosis.

Also anthrax bacilli can be identified by ***immunofluorescence*** assay.

Nucleic acids of *B. anthracis* are determined by ***PCR***.

For ***isolation of the pure culture*** the specimens are inoculated into meat-peptone agar, meat-peptone broth and blood agar (the latter yields non-hemolytic colonies). The isolated culture is differentiated from other bacteria by its morphological, biochemical and antigenic properties.

Laboratory animals (mice, guinea pigs and rabbits) are inoculated directly by pathogenic material or by isolated culture. As an example, *B. anthracis* causes the death of mice in 24-48 hours after inoculation. Microscopical examination of smears made from blood and internal organs reveals anthrax bacilli, which are surrounded by a capsule.

Post-mortem sections as well as leather and hair used as raw materials are examined by ***thermoprecipitin reaction*** (***Ascoli's test***) to detect anthrax antigens.

Bacterial phagotyping is a valuable test, as the specific bacteriophage causes the lysis of pathogenic culture.

In ***serological diagnosis*** various kinds of ELISA are developed to determine antibodies against bacterial toxins, capsular and spore-derived antigens. Acute and convalescent sera obtained 3-4 weeks afterwards should be tested. A positive result is a fourfold rise of specific antibody levels.

Treatment and Prophylaxis of Anthrax

Penicillin is the drug of choice for ***treatment of anthrax***, but it must be started early. Macrolides, fluoroquinolones (e.g., ciprofloxacin), and vancomycin are also active against these bacteria. Treatment may also include passive immunization with anthrax antitoxic immune globulin.

For ***specific prophylaxis*** live (***attenuated***) ***vaccine*** containing spores of non-capsulated *B. anthracis* vaccine strain is used in many countries to immunize herbivores and groups of humans with high occupational risk of infection.

The vaccine is harmless, but with some side effects; it produces the immunity quite rapidly (in 48 hours) and for a period of over a year. It is inoculated in a single dose.

Another anthrax vaccine is an aluminum hydroxide-precipitated protective antigen.

General measures of anthrax control are carried out in tight cooperation with veterinary workers. These measures are aimed for timely recognition, isolation, and treatment of sick animals. They also include thorough disinfection of premises for livestock, affected territory and all the objects, followed by ploughing the pastures. Carcasses of animals died of anthrax must be burnt or buried in specially assigned areas.

PATHOGENIC BRUCELLAE – CAUSATIVE AGENTS OF BRUCELLOSIS

The History of Discovery

In 1886 on the Island of Malta an English bacteriologist D. Bruce demonstrated the presence of the causative agent of Malta fever in the spleen of a dead patient. Later in 1887 he isolated these bacteria in pure culture.

In 1896 the Danish scientist B. Bang established the etiology of contagious abortion of cattle. In 1914 the American investigator G. Traum isolated from pigs the bacteria responsible for contagious abortion of these animals. Other brucellae species were discovered in 1953, 1957, and 1966.

Classification of *Brucellae*

Brucellae belong to the family *Brucellaceae*, genus *Brucella*. For a long time they were classified into numerous species, depending on primarily affected host. Among them are brucellae of goats and sheep – *Brucella melitensis*; brucellae of cattle – *Brucella abortus*; brucellae of pigs – *Brucella suis*; brucellae of forest rats – *Brucella neotomae*; the causative agents of abortion in sheep – *Brucella ovis*; and *B. canis* of dogs.

However, DNA-DNA hybridization studies elaborated in 1980s suggested that *Brucella* is a monospecific genus. These data were confirmed in further studies, and the latest edition of Bergey's Manual of Determinative Bacteriology consolidated all brucellae of medical importance within the only species *Brucella melitensis*.

Nevertheless, isolates from human infection are still classified into groups using the former species names. This correlates with the animal species the strains of brucelllae are predominantly isolated from (cattle, goats, pigs, and dogs.) These groups are differentiated on the basis of their special phenotypical traits.

Human pathology is predominantly associated with *B. melitensis*, *B. abortus*, and *B. suis*.

Structure and Properties of *Brucellae*

Morphology

Isolates of *Brucellae* form small ***gram-negative*** ovoid-shaped coccobacteria. They have no spores or capsules (in some strains of *Brucella melitensis* the capsule is present).

Brucellae have unusual genome structure composed of two non-identical circular closed chromosomes without plasmids.

Cultivation

When recovered from patients, brucellae propagate slowly, being cultured for 1-2 weeks. In laboratory subcultures the growth may appear in 1-2 days. The optimal temperature for culture is 37°C, pH 6.8-7.2.

Brucellae are cultured on special media, e.g. liver-extract agar and liver-extract broth. They produce small, convex, smooth colonies with a white or pearly hue. *Brucella abortus* prefers to grow in atmosphere of 5-10% of carbon dioxide. Selective media containing certain dyes and antibiotics are used for isolating of bacteria. Blood agar culture renders nonhemolytic glistening colonies.

All brucella actively propagate in the yolk sac of chicken embryos.

Biochemical properties

Brucellae are the ***aerobic*** bacteria. They produce catalase and oxidase.

The bacteria display weak carbohydrate fermentation (sometimes metabolize glucose).

Some strains hydrolyze urea and asparagin, reduce nitrates to nitrites, and metabolize proteins, peptones and amino acids with release of ammonia and hydrogen sulfide. The bacteria don’t liquefy gelatin.

Also they produce enzymes lipase, phosphatase, and hyaluronidase.

Antigenic structure

Brucellae are defined to contain two lipopolysaccharide antigens, ***A*** and ***M***. *Brucella melitensis* carries predominantly M fraction, whereas *Brucella abortus* group – A fraction. Superficial L antigen has been demonstrated; it resembles the capsular Vi antigen.

Virulence factors

Despite evident virulence of brucellae strains, their genome doesn’t harbor genetic pathogenicity islands. So the virulence factors of brucellae remain not well-determined.

Brucellae don’t produce soluble toxins.

An ***endotoxin*** is released as a result of disintegration of the bacterial cell, but it poorly activates the innate immune response. It can’t be excluded that it maintains microbial survival inside phagocytes.

In addition, low-molecular weight components of microbial body block phagosome-lysosome fusion.

Microbial ***capsule*** protects brucellae from phagocytosis.

Also the bacteria express ***invasive enzymes***, e.g. hyaluronidase and lipase.

Resistance

Brucellae are characterized by marked resistance and viability. They survive for a long time at low temperatures (up to 4 months in ice). Bacteria live for about 4 months in urine and animal feces, from 3 to 4 months in sheep’s wool and sheep’s cheese, for 1 month in dust, about 20 days in meat, and for 7 days in milk.

Nevertheless, brucellae are sensitive to high temperatures and disinfectants. At 60°C they are destroyed in 30 min, at 80-95° in 5 min, boiling kills them almost instantly.

They are easily inactivated by all conventional disinfectants.

Pathogenesis and Clinical Findings in Brucellosis

Brucellosis is a ***zoonotic*** infection contracted by humans via direct or indirect ***contact*** with animals (***sources of infection***), which were infected (usually chronically) with *Brucella.*

The disease usually affects veterinary and zootechnical personnel, herdsmen, livestock handlers, etc.

Infection can be established via ***cutaneous*** (contact with infected tissues of animals), ***respiratory***, or ***alimentary*** routes. Cheese made from unpasteurized goats’ milk is a particularly common vehicle.

Symptoms of brucellosis are generally non-specific, and the onset of illness may be acute or insidious. As a result of the ***systemic*** nature of brucellosis, almost any organ of the body might be infected.

The ***incubation period*** lasts about 1-6 weeks.

The pathogens spread from the portal of entry via lymphatic vessels and regional lymph nodes to the bloodstream and then to parenchymatous organs. Due to the marked resistance to phagocytosis the bacteria disseminate throughout the body. They stay long within phagosomes without inactivation. The protracted clinical course of brucellosis

maintained by viable bacteria is generally regarded as ***chronic systemic infection*** (sepsis).

Granulomatous nodules and abscesses emerge in lymphatic tissue, liver, spleen, and bone marrow. The lesions contain viable brucellae, located within the infected cells. The invaded pathogen stimulates the reactions of ***delayed*** (cell-mediated) ***hypersensitivity***. Overproduction of cytotoxic molecules enhances tissue damage.

Specific ***granulomas*** consist of mononuclear cells, epithelioid histiocytes and giant cells; active tissue inflammation results in focal necrosis with subsequent gradual fibrosis.

Clinical ***manifestations*** of brucellosis are highly ***variable*** – from faint febrile ilness ("fever of unknown origin") to evident respiratory infection and joint involvement. Various complications (e.g., osteomyelitis or meningitis) occasionally occur.

Usually *B. abortus* causes mild disease without suppurative complications; *B. suis* infection tends to be chronic with suppurative lesions. *B melitensis* infection is more acute and severe.

A long-lasting ***immunity*** both cellular and humoral is acquired following brucellosis, and the patient usually becomes resistant to recurrent infection. ***Cell-mediated reactions*** (T-lymphocyte activation and phagocytosis) play the major role in pathogen elimination.

Laboratory Diagnosis of Brucellosis

The ***specimens*** taken from patient's blood and urine (for isolation of the pathogen), serum (for detection of antibodies), milk and dairy products (for detection of brucellae) are examined. Any suspected *Brucella* isolates determined in the clinical laboratory should be handled in a biological safety cabinet.

PCR with primers specific to various brucella species is used for detection of pathogens directly in clinical specimens.

Blood or tissues samples are inoculated for ***culture*** into liver-extract or ascitic-fluid broth, or trypticase-soy broth. At intervals of several days, subcultures are made on solid media of similar composition. All cultures are incubated in 10% CO_2 and should be observed and subcultured for at least 3 weeks prior to negative conclusion.

The brucellae are aerobic small gram-negative coccobacilli, which are nonhemolytic and oxidase-positive; they do not ferment lactose or glucose.

Most of strains are urease-positive. Bacteria matching the criteria are further tested for agglutination with specific anti-brucella serum.

Suspected *Brucella* isolates should be sent to a reference laboratory for final identification.

Brucella cultures may be isolated by the ***biological method***. To aim this, guinea pigs or mice are injected with test materials. A month later the pure culture is isolated.

Serological tests are the most practically relevant in laboratory diagnosis of brucellosis. They are performed from the 2^{nd} week after the disease onset.

Huddleson reaction or tentative slide agglutination test is usually applied for primary examination of ***specific antibodies*** in brucellosis.

Extended ***tube agglutionation test*** or ***Wright reaction*** validates the primary positive result of afflutination and confirms the diagnosis of brucellosis. Wright's reaction is valued positive in a titer of specific antibodies 1:200 and more.

Allergic skin test (or ***Burne brucellin test***) is used to determine delayed hypersensitivity in brucellosis. Burne test is conducted with specific infectious allergen ***brucellin*** derived from brucella cells. The analysis becomes positive from the 2^{nd}-3^{rd} week of the disease.

Treatment and Prophylaxis of Brucellosis

Because of their intracellular location, brucellae are not readily eradicated by antimicrobial agents. For best results, the ***treatment*** must be prolonged. Administration of aminoglycosides (e.g., gentamycin), fluoroquinolones, and tetracyclines accelerates the recovery.

Chronic cases can be additionally treated with killed vaccine that activates antimicrobial immunity.

For ***specific prophylaxis*** of brucellosis various live and inactivated vaccines were introduced into clinical practice. They can be administered to protect the contact persons as well as the personnel with occupational risk of brucellosis. However, vaccination is not able to confer the long lasting high-grade immunity.

CAUSATIVE AGENT OF TULAREMIA

The History of Discovery

Tularemia causative agent was first described by G. McCoy and Ch. Chapin in Californian town Tulare in 1912. Later it was studied thoroughly by E. Francis; thereby the agent was finally named as *Francisella tularensis*.

Classification

Causative agent of tularemia, *Francisella tularensis*, is placed into the separate family *Francisellaceae* apart from other similar bacteria.

Other species or biogroups of *Francisella* genus may cause human infection, but *F. tularensis* appeared to be the most virulent agent within the genus.

Structure and Properties of *Francisella tularensis*

Morphology

Francisella tularensis is viewed as small (0.2-0.7 μm) pleomorphic ***gram-negative*** coccobacterium. It is a fastidious non-motile, non-spore-forming microorganism.

Within infected tissues *F. tularensis* produces ***capsule***.

Cultivation

The bacteria don't grow in ordinary media. They are cultured on special media with growth factors – glucose cysteine blood agar, glucose blood agar, chocolate agar or charcoal yeast extract agar at 37°C yielding small, smooth, gray-white, flat, and shiny colonies after 48 h of incubation. Increased concentrations of CO_2 stimulate bacterial propagation.

Biological method is also used for microbial cultivation in laboratory animals (mice or guinea pigs).

Biochemical properties

Francisellas are strict ***aerobes***, being catalase-positive, but oxidase-negative.

The bacteria ferment glucose and maltose yielding acid end products. They metabolize asparagin and produce hydrogen sulfide after protein fermenation.

There are two major biogroups or subspecies of *F. tularensis* – type A and type B.

Type A occurs only in North America, is lethal for rabbits, produces severe illness in humans, ferments glycerol, and contains citrulline ureidase enzyme.

Type B lacks these biochemical features, is not lethal for rabbits, produces milder disease in humans. Bacteria of type B are often isolated from rodents or from water in Europe, Asia, and North America.

Antigenic structure

F. tularensis contains somatic lipopolysaccharide ***O-antigen*** and superficial capsule-like ***Vi*** antigen.

Virulence factors

Virulence factors of *F. tularensis* are not completely studied.

These bacteria are not found to produce exotoxins.

An ***endotoxin*** is released after the degradation of microbial cells. As in brucellae, it poorly stimulates innate immune response.

Other bacterial virulent factors (e.g., ***capsule***) inhibit phagocytosis and prevent phagosome-lysosome fusion.

Cell wall allergens stimulate the reactions of delayed hypersensitivity.

Some strains may produce hemolysins.

Francisellas are the ***extremely invasive bacteria***, and they can infect humans even through intact skin.

Resistance

Francisellas are markedly resistant in the environment. They stay viable in water at 4^{o}C for about 4 months, at 20^{o}C – more than 2 month. Nevertheless, francisellas are sensitive to heating at 60-80^{o}C, and commonly used disinfectants readily inactivate them.

Pathogenesis and Clinical Findings in Tularemia

The natural reservoirs and ***sources of infection*** of *F. tularensis* include numerous ***rodent*** species (rats, muskrats, mice, etc.), hares, rabbits and other animals. Humans can become infected after ***direct animal contact*** or via insect bites (ticks, biting flies, mosquitoes, etc.)

F. tularensis is highly invasive: extremely ***low infectious dose*** of 50 microbial cells penetrated through the skin or mucous membranes or even about 10 cells by inhalation is enough to result in infection. In most of cases the bacteria enter the body through skin lesions.

Incubation period is short ranging 2-6 days.

The clinical manifestations of tularemia in human hosts depend on the ***site of entry*** of the bacteria (cutaneous inoculation, inhalation, or ingestion).

The infection has acute onset with chills and fever. Primary ulcerative lesion appears on the skin in the site of entry. The bacteria enter the phagocytes and actively propagate. They show remarkable resistance to microbicidal activities of phagocytes

F. tularensis replicate intracellularly causing cell destruction. Virulence factors of bacteria stimulate apoptosis of infected cells.

F. tularensis spread to regional lymph nodes that enlarge and become painful and necrotic (*primary buboes*). Further the bacteria migrate to organs and tissues. Degradation of bacterial cells leads to endotoxin release. Microbial accumulations in affected tissues stimulate granulomatous inflammation followed by cell-mediated reactions of ***delayed hypersensitivity***.

Inhalation of infective aerosol results in severe pneumonitis.

Clinical forms of tularemia are classified as ***ulceroglandular*** (primarily affecting skin and lymph nodes), ***glandular***, ***conjunctival*** and ***oculoglandular***, ***oropharyngeal***, ***pneumonic***, or systemic ***typhoidal***. Various mixed clinical variations are observed.

Severe pneumonic form of tularemia is manifested like atypical pneumonia with fever, cough with low sputum, chest pain, and ulcerative damage of lymph nodes.

Pneumonic and systemic diseases demonstrate high lethality of 30-60%; the fatality of more common local infections is about 3%.

Due to the high environmental resistance, enhanced invasiveness, minimal infectious dose, and severe course of the disease *F. tularensis* bacteria are accounted as the potential agents of bioterrorist attacks.

They are present in Tier 1 of US Biological Select Agents list with the highest rank of public threat.

Laboratory Diagnosis of Tularemia

Tularemia may be diagnosed by isolation of the bacteria from various ***specimens***: blood, pleural fluid, sputum, lymph nodes, wounds, or gastric aspirates that depend on the clinical form of infection.

In order to avoid laboratory-acquired infection, francisella should be cultured only in biological safety cabinet of BSL-2 level maintaining all personal safety measures.

Laboratory procedures that may result in aerosol production require BSL-3 safety conditions

Microbial ***antigens*** in specimens are detected by immunofluorescence assay and ELISA.

Nucleic acids of bacteria are determined by ***PCR***.

For ***cultivation*** cysteine blood agar or glucose blood agar are used.

More effective is ***biobacteriological*** method, where primary animal infection is followed by further inoculation of animal specimen into nutrient media for culture.

Suspected *F. tularensis* isolates should be delivered to a reference laboratory for confirmatory identification; it is related with the evident danger of laboratory-acquired infection.

In standard laboratory practice the ***diagnosis of tularemia*** relies largely upon the ***serological studies*** of patient's serum for presence of specific antibodies.

Agglutination reaction is regarded as positive in a titer of 1:100-1:200 and more. Paired serum samples demonstrate the elevation of Ab titers. However, cross-reactions of antibodies with *Brucella spp., Yersinia spp.*, etc. have been documented.

Allergic skin test with infectious allergen ***tularin*** is obviously helpful in diagnosis of infection. It evaluates specific cell-mediated response to *F. tularensis* based on delayed hypersensitivity.

Treatment and Prophylaxis of Tularemia

Chemotherapy with aminoglycosides (gentamicin or amikacin) or fluoroquinolones produces rapid clinical improvement. Tetracyclines (doxycycline) are almost equally effective.

For ***specific prophylaxis*** the individuals of high risk (e.g., laboratory personnel) are immunized with live attenuated vaccine of *F. tularensis* created by N. Gaisky and B. Elbert. Protection with live vaccine maintains the specific immunity for several years.

Chapter 10

CAUSATIVE AGENTS OF LEPTOSPIROSIS AND BORRELIOSES

PATHOGENIC LEPTOSPIRAE

The History of Discovery

First observations of icteric leptospirosis with renal failure were registrated in 1886 by A. Weil in Germany, though the similar disorders were described still in ancient ages (e.g., the disease of rice harvesters in China or autumn fever in Japan).

In 1907 A. Simpson discovered the presence of hook-ended spirochetes in the kidney specimen of patient, who supposed to die from yellow fever. He called them *Spirochaeta interrogans*, as they resembled question mark.

Only in 1915 these agents were re-discovered by R. Inada and R. Ido in Japan, who isolated bacteria from the blood of Japanese miners with infectious jaundice, and almost at the same time by two independent groups of German researchers (P. Uhlenhuth and W. Fromme; E. Hubener and H. Reiter) after examination of German soldiers in northeast France that suffered from so-called "French disease" during World War I.

Modern Classification of Leptospirae

Leptospirae pertain to the order *Spirochaetales*, family *Leptospiraceae*, and genus *Leptospira*.

Recently classification of leptospirae has been greatly changed within the borders of the same genus. Before early 90th only two leptospira species have been distinguished, *L. interrogans* that contained all pathogenic serovars, and *L. biflexa*, encompassed all environmental saprophytic strains. Further division was grounded on microbial serologic properties. *L. interrogans* comprised more than 200 serovars, while *L. biflexa* included about 60 serovars. Bacterial serovars were consolidated into various serogroups.

With the progress of bacterial genetic typing, genotypic classification of leptospirae substituted the serological division, and numerous genomospecies accumulated the serovars of initial species, *L. interrogans* and *L. biflexa*.

More than 20 genetic species of leptospirae are distinguished now. In this classification pathogenic and saprophytic serovars can be placed into the same genomospecies. Moreover, former species *L. interrogans sensu lato* and *L. biflexa sensu lato* don't coincide with the same name genomospecies. Therefore, previous phenotypic classification lacked correspondence with modern genetic typing of leptospirae, albeit serological division remains convenient for practical use and retains its value for seroepidemiological studies.

Main genomospecies and serogroups of leptospirae are present in Table 6.

Table 6

Various serogroups of leptospirae, associated with certain genomospecies

Serogroups of leptospirae	**Genomospecies**
Icterohaemorrhagiae, Grippotyphosa, Mini, Pomona, Canicola, Australis, Pyrogenes, Autumnalis, and others	*L. interrogans*
Semaranga, Andamana	*L. biflexa*
Icterohaemorrhagiae, Pyrogenes, Javanica, Mini, Tarassovi, and others	*L. weilii*
Icterohaemorrhagiae, Lyme, Tarassovi, Javanica, Canicola, Panama, and others	*L. inadai*
Pyrogenes, Autumnalis, Australis, Javanica, Tarassovi, Mini, Bataviae, and others	*L borgpetersenii*
Pyrogenes, Australis, Louisiana, Bataviae, Tarassovi, Autumnalis, Pomona, Panama, and others	*L. noguchii*
Icterohaemorrhagiae, Grippotyphosa, Australis, Autumnalis, Pomona, Canicola, and others	*L. kirschneri*
Grippotyphosa, Pyrogenes, Tarassovi, Bataviae, Mini, Autumnalis, Pomona, Javanica, and others	*L. santarosai*
Mini, Javanica, and others	*L. alexanderi*
Mini, Javanica, Semaranga, and others	*L. meyeri*
Codice	*L. wolbachii*
Hurstbridge	*L. fainei*
Turneria	*L. parva*

Structure and properties of leptospirae

Morphology

Leptospirae have very thin cell structure, usually 0.1 by 20 μm in size. These spiral bacteria make multiple turns around the axial filaments, thus forming small primary coils. Being supercoiled, leptospirae produce secondary twists with distinctive hooks at their ends, shaping interrogative mark, or letters C and S under microscopy. The organisms bear two ***axial filaments*** (***periplasmic flagella***) attached at opposite ends to basal bodies within periplasmic space. Flagella ensure striking motility of microbial cells.

The bacterial genome was shown to be composed of two parts: large 4,400 kb chromosome and small 350 kb chromosome. No other plasmids were described.

Leptospirae don't contain spores and capsules. Microbial body is encased within the outer membrane. Bacterial lipopolysaccharide is similar with other spirochetes, but shows lower endotoxin activity.

Leptospirae poorly accept aniline dyes due to their compact structure and large lipid contents. These bacteria are gram-negative; also they stain pinkish with Romanowsky-Giemsa method. The best technique of bacterial visualization is ***dark field microscopy***. Silver impregnation or Burri stain with Indian ink background may be applied as well.

Cultivation

Leptospirae are cumbersome for culture. Optimal growth temperature for cultivation is 28-30°C. Bacteria propagate slowly in liquid and semisolid media, e.g. Vervoort-Wolff, Fletcher, Noguchi and others. The media contain serum or albumin, vitamins, long-chain fatty acids, and ammonium salts. Several synthetic protein-free media were elaborated, e.g. complex oleic acid-albumin medium is used. Primary growth appears in several weeks of cultivation, subcultures grow within 1-2 weeks.

Biochemical properties

Leptospirae are ***obligate aerobic bacteria***. They produce catalase and oxidase.

As many other spirochetes, leptospirae have slow metabolism, which is not completely elucidated. Bacteria use exclusively long-chain fatty acids as the only source of carbon. They can't utilize peptides and carbohydrates as energy supplies. Ammonium salts are used as the source of nitrogen.

Antigenic structure

Antigenic composition of leptospirae is complex and renders great cross-reactivity between various serogroups and serovars.

The outer membrane of bacteria contains LPS antigen and various lipoproteins, i.e. outer membrane proteins (***OMPs***) that show antigenic activity.

Microbial ***LPS*** confers serovar specificity.

Virulence factors

Leptospirae carry various ***adhesins*** promoting microbial attachment to host cells and tissues, e.g. renal epithelial cells.

Bacteria possess low but distinct ***endotoxic activity*** of LPS. Microbial LPS also stimulates platelet aggregation during infection.

Leptospirae are capable of producing several ***hemolysins***, some of them show sphingomyelinase or phospholypase activity. Particular strains elaborate limited number of ***cytotoxins*** of protein or glycolipoprotein nature.

Bacteria express some ***antiphagocytic substances***, and fibronectin-binding protein that hinders microbial opsonization.

Resistance

Leptospirae show resistance to low temperatures, alkaline pH and stay viable in water reservoirs for many months. They are very sensitive to drying and acids. Heating at 56°C for 30 minutes inevitably kills bacteria. Leptospirae are easily lysed in bile-containing media. They are sensitive to standard disinfectants.

Pathogenesis and Clinical Findings in Leptospirosis

Leptospirosis is a typical ***zoonotic disease***. It is regarded as one of the most widespread zoonosis.

The disease is ***transmitted*** predominantly via ***direct contact*** of susceptible host with the urine of infected animal. The incidence of disease is greatly increased in tropical countries with moist climate with maximal incidence in summer or rainy seasons.

The main ***sources of infection*** are ***rodents*** (e.g., rats), which may contract infection to domestic animals (e.g., cattle), dogs, and other mammals. They can appear to be additional infection source for humans.

Leptospirosis is an ***occupational disease***. The increased risk of illness is reported in farmers, fish workers, sewer workers, veterinarians, miners, soldiers and others.

The causative agent usually enters the body through skin lesions or cuts. Also it can penetrate conjunctiva. Long exposure to infected water may provide infection through intact skin. Waterborne and foodborne transmission is possible via contaminated water and foodstuffs. Likewise, inhalation of water aerosol may produce infection.

Incubation period lasts for about 1-2 weeks.

Great number of leptospirosis cases is subclinical or mild. Nevertheless, about 5-10% of patients develop life-threatening icteric leptospirosis with sudden onset, fever, severe headache, transient rashes, myalgia, and abdominal pain. The fever may be biphasic with relapse in 3-4 days. Bacteremia emerges in the first days of illness.

The disease is characterized by profound injury and dysfunction of most inner organs. It ensues from generalized infectious ***vasculitis*** caused by leptospirae that is followed by endothelial damage and tissue inflammation.

Manifested infection results in ***infectious hepatitis*** with jaundice and high serum bilirubin level that maintains for a long time. Up to 40% of affected persons produce acute renal failure due to ***kidney tubular damage***. Necrotizing pancreatitis, lung and cardiac involvement followed by pneumonia and myocarditis are also characteristic for disease. Aseptic meningitis appears in quarter of patients.

If not terminated by efficient treatment, the disease comes into second ***immune phase***. It is followed by bacterial disappearance from the bloodstream with the rise of specific IgM antibodies. Autoimmune mechanisms contribute to disease progression. Various autoantibodies, including anticardiolipin and antineutrophil cytoplasmic antibodies appear in the disease course. Accumulating immune complexes promote complement activation and cell-mediated cytolysis that enhances tissue damage.

Disease lethality in case of icteric leptospirosis varies within 5-15%.

High levels of specific antibodies ultimately cause bacterial elimination that lead to patient recovery. Nevertheless, shedding of viable bacteria with urine is possible long after clinical convalescence.

The disease confers long lasting stable ***immunity***, which is largely maintained by specific antibodies. In most cases the protection is serovar-specific.

Laboratory Diagnosis of Leptospirosis

Specimens are collected from patient's blood, urine, tissue aspirates, or cerebrospinal fluid, which are used for microscopy and culture. Serum is taken for serological tests.

Dark field microscopy can reveal about 10^4 leptospirae/ml. Sample centrifugation can increase the sensitivity of test. Immunofluorescence technique or light microscopy with Giemsa stain are also used to visualize leptospirae in blood or urine. Microscopy of blood can be positive only at the first few days of the disease in bacteremia stage.

Serologic determination of leptospiral antigens in clinical specimens by ELISA provides higher sensitivity and accuracy comparing with microscopic methods.

Bacterial culture is difficult in routine practice. Patient's blood should be taken only within first days of the disease in bacteremia stage. Urine is tested from the second week of the disease onset.

Samples are inoculated into special nutrient media. Cultures are tested weekly by dark field microscopy for up to 13 weeks. Identification of bacteria is improved by serological methods or molecular tests (PCR). Faster detection of leptospiral growth is possible by radiometric methods.

To accelerate microbial isolation intraperitoneal inoculation of hamsters or guinea pigs with patient's material is elaborated. Leptospirae can be detected in peritoneal cavity of infected animals at the end of the first week after inoculation.

Serological tests prevail in laboratory diagnosis of leptospirosis. Various methods are applied to clinical practice.

In case of ***microscopic agglutination test*** (***MAT***) patient's serum containing specific antibodies is incubated with antigenic mixture of various live leptospiral serovars. After incubation the reaction is evaluated mainly by dark field microscopy. The titer or end point of the reaction is the highest dilution of serum, where 50% agglutination of leptospirae occurs. Antibody titers of 1/200-1/400 are regarded as the positive result. Acute infection elicits much more high titers of specific antibodies (even greater than 1/25,000).

Complement fixation test, indirect hemagglutination and ELISA are also used for serological diagnosis.

Molecular methods, including DNA and RNA ***hybridization*** and ***PCR*** show highest sensitivity but can't determine the serovar of isolated bacteria.

Specific Prophylaxis and Treatment of Leptospirosis

Early vaccines for ***specific prophylaxis*** of leptospirosis contained the mixture of inactivated leptospirae cultured in serum media. After injection they provoked various side effects. Modern vaccines are obtained from serum-free media and include a number of the most clinically significant serovars (*L. icterohaemorrhagiae*, *L. canicola*, *L. grippotyphosa*, and others). These biological products can be used for vaccination of domestic animals as well as for protection of humans from groups of risk.

Antibiotic ***treatment*** of the disease should be started as soon as possible. Beta-lactams (amoxycillin, cephalosporins) and doxycycline are regarded as the most effective drugs for leptospirosis treatment. Patients with acute renal failure require urgent hemodialysis.

PATHOGENIC BORRELIA – CAUSATIVE AGENTS OF RELAPSING FEVERS AND LYME DISEASE

The History of Discovery

Borrelia recurrentis, a causative agent of human ***epidemic relapsing fever***, was discovered by O. Obermeier in 1868. The causative agents of similar zoonotic diseases, i.e. ***endemic relapsing fevers***, were discovered later (e.g., *B. duttoni*, *B. persica*, *B. caucasica* and others).

And in 1982 W. Burgdorfer isolated one more serious borrelia pathogen that caused systemic tick-borne borreliosis in animals and humans. This agent is known now as *B. burgdorferi*. The disease was first described in Lyme town, USA in 1977, and thereby was entitled as ***Lyme disease***.

Classification of Borrelia

All pathogenic borrelia pertain to the order *Spirochaetales*, family *Spirochaetaceae*, and genus *Borrelia*. It was already mentioned that *Borrelia recurrentis* causes ***epidemic relapsing fever***, while *B. duttoni*, *B. persica*, *B. caucasica*, *B. hispanica* and others are the causative agents of tick-born ***endemic relapsing fevers***.

Another representative of *Borrelia* genus, *B. burgdorferi*, was still recently regarded as homogenous microbial species. However, molecular genotyping of numerous *B. burgdorferi* isolates revealed that these bacteria are enough divergent and pertain to closely related but distinct species. It has become evident that former *B. burgdorferi* microbial cluster, termed now as *B. burgdorferi sensu lato*, is referred to all *B. burgdorferi* isolates that pertain to various novel borrelial species.

Today more than 15 definite species are distinguished within *B. burgdorferi sensu lato* genogroup. Among them the members of 3 species, *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* were proven to cause ***Lyme borrelliosis*** in humans.

Structure and Common Properties of Borreliae

Morphology

Borreliae are thin long spiral-shaped ***gram-negative*** bacteria with pointed ends. Their size varies in the range 0.2-0.4 μm in width and 10-30 μm in length. Bacterial cell forms large, obtuse-angled, irregular coils of total number from 3 to 10. Cytoplasmatic cylinder of microbial body is covered by outer membrane similar with other spirochetes. Bacterial endoflagella (7-11 for *B. burgdorferi*) are located within periplasmic space being attached to the opposite ends of microbial cell. Armed with endoflagella, borreliae exhibit striking motility.

Sequenced genome of *B. burgdorferi* is composed of 910 kb linear chromosome and various circular and linear plasmids.

Borreliae easily accept aniline dyes due to high nucleoprotein content and stain ***blue-violet*** with ***Romanowsky-Giemsa stain***. Also spirochetes can be detected by dark field or fluorescent microscopy.

Cultivation

B. recurrentis grow under anaerobic conditions in liquid media enriched with ascitic fluid, blood, serum, or pieces of tissues and organs but the bacteria alter their virulence during culturing. They easily propagate, when inoculated onto the chorioallantoic membrane of chicken embryo.

B. burgdorferi sensu lato is cultured in commercially available complex ***Barbour-Stoenner-Kelly*** (or ***BSK***) medium supplemented with amino acids, vitamins, yeast extract and rifampicin, phosphomycin, and amphotericin B antibiotics to inhibit concomitant flora. The primary growth may be obtained at 1-2 week of incubation.

Biochemical properties

Borreliae are fastidious microorganisms that need multiple growth factors. They are determined to be non-fermentative bacteria. Their metabolism is slow and needs further elucidation.

Antigenic structure

B. burgdorferi contains various antigens of lipid, lipoprotein, and carbohydrate nature. Most important are the ***outer surface proteins OspA-OspG***, ***heat shock proteins*** (***Hsp***) and flagellin from bacterial endoflagella.

OspC lipoprotein shows remarkable antigenic variability.

Antigens of *B. recurrentis* are similar with above mentioned but they are not completely defined yet. These bacteria considerably change their antigenic composition under permanent pressure of host immune system during infection course.

Virulence factors

Bacterial lipopolysaccharides and lipoproteins mediate ***endotoxin activity*** of borreliae. Multiple ***adhesins*** promote microbial attachment to the host cells. Microbial ***heat shock proteins*** activate massive proinflammatory cytokine release by host immune cells that is followed by tissue damage.

Resistance

Bacteria demonstrate relatively low resistance, but they can withstand deep freezing for 4-5 days. Also they stay viable for several days in liquid media at room temperature. Heating at 45°C kills them in 30 minutes. Borreliae are easily inactivated by ordinary disinfectants.

Pathogenesis and Clinical Findings in Epidemic and Endemic Relapsing Fevers

Epidemic relapsing fever is an acute ***anthroponotic vector-borne disease*** transmitted by ***louse*** *Pediculus humanus*.

The ***human*** is the only ***source of infection***. The louse becomes infectious sucking the patient's blood. After 5-12 days of microbial propagation within louse body it starts to transfer disease to susceptible persons. Lice contain the pathogen during the whole life cycle but they are lack of transovarial transmission of borreliae.

Humans acquire infection scratching the site of louse bite, thus crushing the louse and rubbing the parasite's hemolymph with borreliae into injured skin.

Incubation period lasts 3 to 10 days. After primary inoculation bacteria propagate within phagocytes, endothelial cells and parenchymatous organs, e.g. spleen, liver and kidneys.

Multiplied borreliae come into the bloodstream. This is followed by microbial lysis due to complement activation and cytotoxic activity of immune cells. Microbial destruction leads to ***endotoxin release*** that provokes sudden disease onset with high fever, chills, and headache.

Toxic action of microbial substances damages the cells of blood vessels and most of inner organs thereby producing spleen and liver necroses, kidney hemorrhagic lesions, platelet aggregation, etc.

The severity of the illness gradually decreases following borrelia elimination from the bloodstream. The remained microbials hide within host cells and continue propagation. Subsequent ***relapse of the disease*** occurs in 5-7 days resulting from the ***next emergence of borreliae*** in the bloodstream.

Latest generations of bacteria alter their antigenicity under the selective pressure of immune response thereby evading host defense. They provoke ***3-5 sequential attacks*** of the disease. Nevertheless, the rise of specific antibodies and phagocytic activity eventually cause bacterial elimination. Every next relapse of fever is milder, and the interval between attacks increases. The absence of fever within 25 days confirms patient's recovery.

Immunity in epidemic relapsing fever is largely humoral and not very stable.

Endemic relapsing fever is ***zoonotic*** disease, caused by numerous endemic borrelia species, e.g. *B. duttoni*, *B. persica*, *B. hispanica*, *B. caucasica* and others. It is similar with human relapsing fever but not as sharp as the human illness. The disease has seasonal prevalence with the highest incidence in spring and summer.

Rodents are the predominant ***sources of infection***. The main ***vectors*** for disease transmission are the ***ticks*** of genus *Ornithodorus*. Transovarial transmission of borreliae in ticks is common.

Humans are infected via tick bites or by rubbing of crushed parasite. The ***incubation period*** of disorder is about 5-10 days. The specific papule or *primary affect* appears at the bite site.

Pathogenesis and clinical findings in endemic disease resemble epidemic relapsing fever.

The ***immunity*** against tick-borne fever is widespread in endemic regions. Thus, the newcomers devoid of specific acquired immunity are the most affected persons.

Laboratory Diagnosis of Relapsing Fevers

Patient's blood is collected during the febrile period of disease.

Microscopy of thick blood smears is used to detect borreliae in the drop of patient's blood. Specimens are stained by Romanowsky-Giemsa or examined by dark field microscopy.

Various ***serological tests*** are applied to determine specific ***anti-borrelial antibodies*** especially in the period of apyrexia. Complement fixation test or reactions of specific borrelia immobilization by antibodies may be employed.

PCR is elaborated as rapid method for microbial DNA detection.

To discriminate endemic tick-born relapsing fever from human epidemic disease the ***biological method*** of ***experimental animal infection*** is used. Mice, rats or guinea pigs are inoculated with infected blood of sick person. In case of endemic fever the animals develop the disease after short incubation period, and spirochetes can be readily detected in blood of infected animal by microscopy.

Treatment and Prophylaxis of Relapsing Fevers

Antibiotic ***treatment*** with drugs of various groups (e.g., penicillins, tetracyclines and macrolides) is effective in relapsing fevers.

The ***measures of non-specific protection*** are prevalent in disease prophylaxis, specific vaccines are not available.

Pathogenesis and Clinical Findings in Lyme Disease

Lyme disease is a polyorganic and multistage ***zoonotic*** infection. The disease is transmitted by ***ticks*** of definite species from *I. ricinus* complex. *I. scapularis* and *I. pacificus* predominantly spread the disease in Northern America, while *I. ricinus* and *I. persulcatus* are the predominant vectors in Europe and Asia.

The main ***sources of infection*** are rodents, birds, wild and domestic animals (e.g., deer, cattle, sheep, dogs and many others.)

Usually humans are infected with *B. burgdorferi* ***via tick bites***.

Clinical picture of Lyme disease develops in several stages, the pathogen affects a variety of tissues and organs, including skin, joints, heart, and nervous system.

Early local infection flares up by ***primary erythema migrans***, an annular skin rash that begins days to weeks after a tick bite.

Hematogenous dissemination of spirochetes during the ***second stage*** (known as ***early dissiminated infection***) over the next days or weeks results in multiple skin lesions (*secondary erythema migrans*), as well as meningitis, radiculoneuritis, atrio-ventricular block, myocarditis, and oligoarticular arthritis. Borreliae interact with endothelial cells, synovial tissue, glial cells of CNS, promoting systemic inflammation.

Persistent infection at ***third stage*** (***late Lyme borreliosis***) occurs months to years after the initial exposure and can be associated with *acrodermatitis chronica atrophicans*, encephalomyelitis with encephalopathy, and persistent arthritis.

Now it is almost proven that various symptoms and complications of Lyme disease result from human infection by certain genospecies of borrelia. For instance, Lyme arthritis is associated with infection of *B. burgdorferi sensu stricto*; neuroborreliosis depends on *B. garinii* infection whereas acrodermatitis chronica atrophicans is related with *B. afzelii*.

At the latest stages the disease progression is maintained by ***multiple autoimmune reactions*** evolved in the pathology course.

When untreated, the disease can lead to deep disability of patient or even cause patient death.

Laboratory Diagnosis of Lyme Disease

Lyme borreliosis is largely a clinical diagnosis; laboratory testing is used to confirm clinical findings.

Serological methods are most feasible for laboratory diagnosis of Lyme disease detecting ***specific antibodies*** against the causative agent. They include indirect immunofluorescence assays, enzyme-linked immunosorbent assays (ELISA), and immunoblotting assay.

In USA two-step procedure is recommended for serological diagnosis of Lyme disease. The first step employs a sensitive serological test, such as ELISA. Specimens found to be negative are not tested further. All the specimens with positive or equivocal results are tested by immunoblotting.

In Europe and Asia, the elaboration of a uniform approach for the immunoserologic evaluation of the disease is complicated by the presence of bacteria from three genospecies of *B. burgdorferi sensu lato* genogroup and by significant antigenic variation within each genospecies.

For ***culture isolation*** of *B. burgdorferi* patient blood, skin biopsy or cutaneous lavage from erythema migrans are used. Culturing is produced in ***BSK medium***. Spirochetes are detected by dark field microscopy or by fluorescent microscopy wiih acridine orange stain.

Molecular methods primarily imply ***PCR*** with specific primers. Patient's blood, synovial or cerebrospinal fluids are tested.

Treatment and Prophylaxis of Lyme Disease

For ***treatment*** of Lyme borreliosis beta-lactam antibiotics (e.g., amoxycillin) as well as doxycycline or azalides (azythromycine) are successfully used. Timely administered antimicrobial treatment prevents the progression of the disease and ensures recovery.

Amoxycillin and doxycycline are also recommended for ***post-exposure chemoprophylaxis*** of Lyme borreliosis after accidental bite of infected tick. If administered within 3-5 days after the exposure, antibiotics completely prevent the disease onset.

For ***specific prophylaxis*** various vaccines, including whole-cell vaccine, live attenuated vaccine and recombinant vaccine based on genetic engineered OspA and OspC proteins are being worked out. The efficacy of OspA vaccine is reported to be about 75-90%.

Chapter 11
CAUSATIVE AGENTS OF RICKKETSIOSES AND Q FEVER

PATHOGENIC RICKETTSIAE

The History of Discovery

First description of pathogenic rickettsiae was made in 1910 by American scientists H. Ricketts and R. Wilder, who revealed small oval-shaped bacteria both in blood of patients with Mexican typhus and in lice, inhabiting patient's body. Later in 1913, Czech microbiologist S. Prowazek discovered the similar agents in blood of patients with typhus fever.

Finally, in 1916 Brazilian pathologist H. da Rocha-Lima, who has been working in close collaboration with S. Prowazek, thoroughly investigated the newly discovered bacteriae, revised all collected data and finally established these organisms as the causative agents of epidemic typhus. He termed them *Rickettsia provazekii* in honor of H. Ricketts and S. Prowazek, who both died, investigating this life-threatening disease.

Further it was found that rickettsioses comprise a numerous group of arthropod-borne diseases. Today they are regarded as the emerging diseases because of 19 currently known rickettsioses more than 10 were discovered in last 20-25 years.

Modern Classification of Rickettsiae

Until quite recently the family *Rickettsiaceae* encompassed a great variety of pathogenic bacteria. Later many of them were organized into separate taxa, which became the members of new bacterial families and even classes.

According to current classification the order *Rickettsiales* comprises two families with pathogenic bacterial representatives: *Rickettsiaceae* and *Anaplasmataceae*

The family *Rickettsiaceae* encompasses the genus *Rickettsia* (with human and animal pathogenic species *R. prowazekii*, *R. typhi*, *R. conorii*, *R. sibirica*, *R. akari* and many others) and genus *Orientia* (species *O. tsutsugamushi*).

The family *Anaplasmataceae* includes the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*.

Only the members of the first family are regarded now as the causative agents of ***rickettsioses***.

Former representatives of *Rickettsiales* order, namely bartonellae and coxiellae, are placed now into the same name new families, *Bartonellaceae* and *Coxiellaceae*.

The main rickettsial pathogens and their diseases are listed in Table 7.

Table 7
Main rickettsioses and their causative agents

Group	Causative agent	The disease	Vector
Typhus group	*R. provazekii*	Epidemic typhus and Brill-Zinsser disease	Human body louse (*Pediculus humanus*)
	R. typhi	Murine typhus or endemic typhus	Several flea species, rarely lice, mites and ticks
Spotted fever group (about 20 known species)	*R. rickettsii*	Rocky Mountain spotted fever (RMSF)	Ticks of genera *Dermacentor, Haemaphysalis* and *Ixodes*
	R. conorii	Mediterranean spotted fever (MSF)	Ticks of genera *Rhipicephalus* and *Haemaphysalis*
	R. sibirica	Siberian tick typhus	Ticks of genera *Dermacentor* and *Haemaphysalis*
	R. africae	African tick bite fever	Ticks of genus *Amblyomma*
	R. australis	Queensland tick typhus	Ticks of genus *Ixodes*
	R. akari	Rickettsialpox	Mite *Allodermanyssus sanguineus*
	R. japonica	Japanese fever	Ticks of genera *Dermacentor, Haemaphysalis* and *Ixodes*
	R. honei	Flinders Island spotted fever	Ticks
	R. felis	Californian flea rickettsioses	Cat fleas
	Astrakhan fever rickettsia	Astrakhan fever	Ticks of genus *Rhipicephalus*
	Israeli tick typhus rickettsia	Israeli spotted fever	Ticks of genus *Rhipicephalus*
Scrub typhus group	Orientia tsutsugamishi	Scrub typhus	Mites of genus *Trombicula*

Structure and Properties of Rickettsiae

Morphology

Rickettsiae are pleomorphic ***gram-negative*** bacteria. Coccoid forms are about 0.5 μm in size. Rod-shaped rickettsiae also demonstrate substantial polymorphism; short organisms of 1 by 1.5 μm as well as long or curved thin rods 3-4 μm in size occur. The thread-like or filamentous forms are up to 40 μm in length.

Rickettsiae are non-motile bacteria; they don't contain spores and capsules. *R. provazekii* may produce capsule-like substance.

These bacteria are visualized by *Romanowsky-Giemsa* stain, *Gimenez* stain (applies fuchsin and malachite green dye for counterstain) and by modified Ziehl-Neelsen stain (*Zdrodovsky method*).

Rickettsial genome is composed of small single circular chromosome 1,000-1,600 kb in size.

Cultivation

All rickettsiae are ***obilgate intracellular parasites***.

Typhus group (***TG***) rickettsiae are localized exclusively in the cytoplasm of affected cells, while ***spotted fever group*** (***SFG***) rickettsiae can invade the cell nuclei, as they possess intracellular motility due to cellular actin polymerization.

For primary isolation various ***cell cultures*** both of tick and mammalian origin are used. Bacterial generation time is about 8-10 hours. Less fastidious is the cultivation of rickettsiae in ***yolk sacs*** of embryonated eggs. For ***animal inoculation*** guinea pigs, rats and mice are used.

Biochemical properties

Rickettsiae metabolism largely depends on cellular biochemical pathways, e.g., the bacteria can't synthesize proteins. For energy gain they possess the enzymes ATP translocases that deliver ATP molecules directly from the infected cells. Also rickettsiae may acquire ATP by oxidative phosphorylation.

Rickettsiae are capable of producing gram-negative cell wall that is composed of peptidoglycan with muramic and diaminopimelic acids.

Antigenic structure

As an example, *R. provazekii* contains specific superficial protein antigens – ***outer membrane proteins OmpA*** and ***OmpB***, and cell wall heat

stable polysaccharide antigen, common for *R. provazekii*, *R. typhi* and certain strains of enterobacterial member, *Proteus* OX-19.

Virulence factors

Rickettsiae contain polysaccharide heat stable ***endotoxin*** and heat labile ***protein toxic substance*** tightly associated with microbial body. The latter can be transformed into toxoid by formaldehyde treatment.

In the course of infection the main deleterious effects of rickettsiae are related with their active propagation inside the infected cells, followed by cell destruction and severe inflammatory response.

Resistance

In normal conditions rickettsiae can survive only in the body of infected host, vector or microbial reservoir. They rapidly lose viability in the natural environment. Dried bacteria usually stay viable for about 5-6 days.

Treatment with ordinary disinfectants and as well as heating at 80^{o}C destroys rickettsiae within several minutes, heating at 100^{o}C cause immediate microbial death.

Pathogenesis and Clinical Findings in Rickettsioses

Rickettsial diseases of the same clinical group show certain similarity in pathogenesis and clinical features. All rickettsioses are characterized by high ***fever***, skin ***rashes***, and generalized ***vasculitis***.

Most severe disorder, ***epidemic typhus***, is caused by *R. provazekii* and transmitted by body louse.

Epidemic typhus is the ***anthroponotic disease***, which follows social disasters (wars, starvation, socioeconomic disorganization with substantial lack of hygienic conditions, etc.) It is believed that epidemic typhus has caused even more deaths than all the wars in human history.

Humans are the main ***sources of infection***. The patients are contagious at fever period and within the week after. The persons, recovered from typhus, retain some viable bacteria for the whole life, thus maintaining the persistent infection.

Feeding ***louse*** becomes infected and within 4-5 days can spread the disease. Nevertheless, lice are lack of transovarial transmission of rickettsiae.

Incubation period of epidemic typhus is about 2 weeks (6 to 24 days) after primary inoculation.

Humans acquire infection rubbing louse excrements containing rickettsiae into injured skin after louse bite.

Rickettsiae multiply primarily in the site of penetration. Then they reach regional lymph nodes and enter the bloodstream, where the bacteria invade endothelial cells. Rickettsiae propagate within cytoplasm of endotheliocytes thereby causing their damage and lysis. The progressing ***endovasculitis*** is manifested by high fever, generalized roseolous-petechial skin rashes, myalgias, pneumonias and severe disorders of central nervous system with headaches, brain sinus thromboses and mental abnormalities (status typhosus). Disseminated intravascular coagulation (***DIC***) may occur.

When untreated, the disease fatality is about 10-30% at the peak of the infection.

During convalescence the growing specific antibodies eliminate bacteria. The disease confers long-lasting ***immunity***.

However, rickettsiae can stay viable within phagocytes for many years, thus provoking the *relapse of epidemic typhus* (or ***Brill-Zinsser disease***) in elderly persons.

Endemic or ***murine typhus*** is similar but much milder ***zoonotic rickettsial disease***, caused by *R. typhi*. Ubiquitous rats and mice are the main reservoirs and sources of this infection in nature. The disease is transmitted by various ***arthropod vectors*** (several flea species, lice, mites, and ticks). In fleas transovarial transmission is possible.

Occasional disease acquisition by humans occurs via contamination of the injured skin, conjunctivae or respiratory tract by aerosols with infectious material, e.g. infected flea feces. Patients can develop fever, headaches, and rash.

The infection confers a relatively ***stable immunity***, cross-reactive with epidemic typhus agent.

Scrub typhus is caused by *Orientia tsutsugamishi*, and transmitted by mites of genus *Trombicula*. It is found in Asia, including India and Japan, and in northern Australia.

The disease is generally similar with epidemic typhus, but the patients reveal the eschar in the primary site of mite bite followed by progressing lymphadenopathy and lymphocytosis.

Rickettsioses from the ***spotted fever group*** comprise a large number of fever diseases. Among them are ***Rocky Mountain spotted fever*** (***RMSF***), caused by *R. rickettsii*, ***Mediterranean spotted fever*** (***MSF***), produced by

R. conorii, ***Siberian tick typhus*** (by *R. sibirica*), ***rickettsialpox*** (by *R. akari*) and several newly described diseases such as ***Japanese fever*** (by *R. japonica*), ***Astrakhan fever*** (Astrakhan fever rickettsia), ***African tick bite fever*** (*R. africae*), ***Israeli spotted fever*** (Israeli tick typhus rickettsia) and some others.

Spotted fevers are largely ***endemic diseases***, transmitted by numerous ***arthropod*** species (ticks, mites, fleas, etc).

Rodents are the main ***sources of infection***.

These disorders are generally characterized by fever, headache, rash, and eschar; the latter appears in the most of the diseases.

The particular diseases (e.g., RMSF) can cause large local outbreaks with high lethality (>30-40%).

Laboratory Diagnosis of Epidemic Typhus and Other Rickettsioses

Specimens are collected from patient's blood, tissue biopsies and autopsy material. Serum is taken for serological tests.

Serological tests are most available for laboratory diagnosis of epidemic typhus and other rickettsioses. Immune fluorescent technique, complement fixation test and ELISA are commonly used. Diagnostic titer of antibodies in epidemic typhus determined by complement fixation test is 1/160 and higher.

By means of serological methods the differential diagnosis between primary epidemic typhus and relapsing Brill-Zinsser disease is possible. The reaction is made with two samples of titrated patient serum, where one sample was previously treated with potent reductive agent, e.g. cystein. Primary epidemic typhus is characterized by high levels of specific serum IgM that are noticeably susceptible to chemical reduction because of large cystin content. Fall of serum antibody titer verifies the presence of specific IgM and confirms the diagnosis of primary epidemic typhus whereas the lack of changes in antibody titers reveals the presence of IgG class antibodies testifying the diagnosis of epidemic typhus relapse, or Brill-Zinsser disease.

Direct detection of rickettsiae in biopsy specimens and arthropod material is possible by immunofluorescence or molecular tests, e.g. ***PCR***.

For culture the specimens are inoculated into ***yolk sacs*** of embryonated eggs, various ***cell lines*** of tick or mammalian origin, or into susceptible ***animal***s, e.g. guinea pigs, mice, etc. Isolation is made only in reference laboratories with appropriate biosafety level.

Inoculation of male guinea pigs with patient's blood makes possible to discriminate endemic typhus from epidemic disease. Once infected with *R. typhi,* animals display specific *periorchitis* (the scrotal swelling) due to rickettsial propagation in the coats of guinea pig testis.

Specific Prophylaxis and Treatment of Epidemic Typhus and Other Rickettsioses

Single dose of 200 mg of ***doxycycline*** is effective for prevention of epidemic fever, thus any suspected case should be treated immediately. Fluoroquinolones are administered in spotted fevers. Treatment with chloramphenicol and macrolides is also possible.

Nevertheless, rickettsiae have intrinsic resistance to β-lactams and aminoglycosides and low sensitivity to sulfonamides.

Various live, formaldehyde-treated, and chemical vaccines are used for ***specific prophylaxis*** of epidemic typhus and other rickettsioses in the centres of disease outbreak or epidemic.

COXIELLA BURNETII – CAUSATIVE AGENT OF Q FEVER

The History of Discovery

For a long time Q fever disease was regarded as rickettsiosis. First description of febrile disorder regularly occurred among abattoirs was made in 1937 by E.H. Derrick in Queensland, Australia. Derrick termed it ***Q fever*** (short for "***query fever***"). He was not successful in isolation of its putative causative agent and supposed it to be the unknown virus. Then M. Burnet and M. Freeman reproduced the disease in animals and detected the infectious agent in the vacuoles of infected cells.

Similar work was produced by G. Davis and H.R. Cox in USA, who isolated rickettsia-like causative agent from patients with unusual fever or Nine Mile disease.

Further collaborative studies proved these newly discovered diseases to be identical. The agent was finally termed as *Coxiella burnetii* – a novel genus within *Rickettsiacea* family.

Only latest phylogenetic investigations relied upon 16S rRNA typing demonstrated considerable divergence between coxiellae and rickettsiae. Today *C. burnetii* is placed into new separate family in the borders of another microbial class lying apart from rickettsiae.

Classification of Q Fever Agent

Q fever agent pertains to the order *Legionellales*, family *Coxiellaciae*, genus *Coxiella*, and species *C. burnetii*.

Structure and Properties of Coxiellae

Morphology

Coxiellae, similar to rickettsiae, are 0.3 by 0.5-1 μm small size gram-negative bacteria with ***obligate intracellular parasitism***. They are poorly stained with Gram method but can be readily detected by *Gimenez* stain as well as by *Zdrodovsky* method.

Two main morphological types of bacteria arise in the course of *C. burnetii* infection.

"***Small-cell***" variant is an extracellular resistant bacterial form with slow metabolism that is capable of invading mammalian phagocytes.

Further it turns into active intracellular "***large-cell***" form within host phagolysosomes. Both types can multiply by binary fission. In unfavorable conditions (e.g., within phagolysosome) large-cell variants can undergo further transformation into ***spore-like*** microbial bodies. The latter are the special bacterial forms resistant to external impacts. Finally, spore-like bodies transform back into small-cell microbials, which spread outside via exocytosis or after lysis of the infected cell.

Bacterial genome carries nucleoid and a number of plasmids.

Cultivation

Coxiellae can't grow in artificial nutrient media. They may be cultured in various ***cell lines*** (e.g., human embryo fibroblasts), embryonated eggs and in susceptible animals (guinea pigs, mice, etc). In cell lines the bacteria are detected in 5-7 days after primary inoculation.

During persistent infection bacteria show slow propagation with doubling time of about 20 h.

Biochemical properties

Coxiellae are more biochemically active than rickettsiae; bacteria use their own methabolic pathways for ATP and protein synthesis.

Antigenic structure

Antigenic composition of coxiellae depends on ***phase variation*** of cellular lipopolysaccharide structure that results from the cascade of mutational events.

Bacteria with LPS of phase I are isolated from infected animals or humans and regarded as highly infectious bacterial form. They are similar with S forms of other microorganisms. Phase II bacteria appear after multiple passages of coxiellae in cell cultures or embrionated eggs. They are related with rough LPS with altered structure.

Virulence factors

Toxic and immunosuppressive factors of *C. burnetii* are not well defined yet. The bacteria produce catalase and superoxide dismutase that inhibit respiratory burst in phagocytes.

After degradation microbial cells release ***endotoxin***.

Resistance

Coxiellae show high resistance in natural environment. The organisms stay viable about 1 year at 4^{o}C. Heating at 70-90^{o}C only partially inactivates bacteria, while 100^{o}C heating kills them within 10 minutes.

They resist desiccation, as well as low and high pH conditions.

Disinfectants work slowly against coxiellae; the bacteria can withstand the action of formaldehyde and carbolic acid but show sensitivity to alcohols and ether.

Pathogenesis and Clinical Findings in Q fever

Q fever is a ***zoonotic*** ubiquitous disease that affects various mammals, birds and arthropods (ticks). Ticks maintain transovarial transmission of coxiellae.

The main ***sources of infection*** for humans are domestic animals and pets (cattle, goats, sheep, cats, dogs, etc.) The infected animals excrete bacteria with urine and feces. The disease is regarded now as occupational hazard in staff working with domestic animals.

The disease is ***transmitted*** predominantly by ***airborne*** (***aerosol***) route after inhalation of dust from contaminated fomites. Fecal-oral transmission is seldom; it occurs mostly after drinking raw milk. Other variants of disease contraction are extremely rare.

Incubation period of illness lasts for about 2-3 weeks.

Coxiellae can persist only ***within phagocytes*** of the host.

Phase I bacteria enter human phagocytes via membrane integrins. After internalization they appear within phagolysosomes that results in large ***vacuole*** formation. Bacteria are extremely resistant to acidic pH 4.7-5.2 of phagolysosomes thereby maintaining their capability to multiply within phagocytes (***incomplete phagocytosis***).

Coxiellae can modulate host immune response. They block reactive oxygen species of phagocytes producing catalase and superoxide dismutase. Bacteria ***depress T cell response*** and cause T helper lymphopenia partially via induction of suppressive cytokine synthesis by host immune cells. On the other hand, they provoke body inflammation stimulating the synthesis of TNF-α and γ-interferon. The cell-mediated reactions of ***delayed hypersensitivity*** are common.

About 60% of disease cases are asymptomatic or may develop mild symptoms. Nevertheless, the rest of cases are severe, especially in immunocompromised patients.

Acute Q fever is characterized by sudden onset with high fever, chills and headaches. Two main clinical syndromes (severe atypical pneumonia and granulomatous hepatitis) are usually common depending predominantly on aerosol or foodborne microbial transmission. Myocarditis and meningoencephalitis may rarely occur.

Chronic Q fever is developed in 6 month after primary infection. It is a potentially fatal disease that is manifested by life-threatening endocarditis and inflammatory syndrome. The endocarditis appears to be highly resistant to antimicrobial therapy.

Post-infectious immunity is rather stable, cellular reactions play predominant role in body protection.

Laboratory Diagnosis of Q fever

Specimens are collected from patient's blood and tissue biopsies. Serum is used for serological examination.

To determine bacteria in tissue specimens indirect immunofluorescence technique is applied.

Serological testing dominates in routine laboratory practice to confirm Q fever diagnosis.

Indirect immunofluorescence and complement fixation test are most widely used. Antibodies against the microbial antigens of both I and II phase are determined. The rise of antibody IgG titer against phase II antigens is characteristic for acute Q fever, while antibodies against the antigens of phase I prevail in chronic disease.

Microagglutination test, dot immunoblotting and ELISA are also available.

Cultivation of coxiellae as well as ***animal experimental infection*** is possible only in specialized laboratories of biosafety level 3 due to high infectivity of Q fever causative agent. The material is inoculated into various cell lines, embryonated eggs and susceptible animals (guinea pigs or mice).

Molecular methods, including ***PCR***, are progressively introduced now into laboratory practice to diagnose Q fever.

Prophylaxis and Treatment of Q fever

C. burnetii is primarily resistant to beta-lactam and aminoglycoside antibiotics. Therefore, macrolides, doxycycline and fluoroquinolones are the drugs of choice for ***treatment*** of acute Q fever.

It is much more difficult to cure chronic Q fever endocarditis. Treatment schemes that include combined therapy with doxycycline and fluoroquinolones at least for 1-3 years are introduced into clinical practice.

For ***specific prophylaxis*** of Q fever various live, formaldehyde-treated, and chemical vaccines were proposed. They are used to protect the persons with occupation of risk. Nevertheless, these vaccines develop various adverse effects and usually confer the immunity of short duration.

Chapter 12
INFECTION-ASSOCIATED DISEASES OF ORAL CAVITY
(***For students of Dentistry faculty***)

Brief Outline of Teeth and Mouth Pathology of Infectious Origin

Now it is generally ascertained that caries, pulpitis, or periodontal diseases are the ailments *essentially related with infection*. They are caused by microbial pathogens has been found in dental plaque.

There are two alternative albeit complementary assumptions concerning the role of dental plaque microflora in pathogenesis of oral disorders.

One of these hypotheses (the hypothesis of *"specific dental plaque"*) presumes the active participation of only limited (or "*specific*") number of bacterial species in the emergence of teeth and mouse pathology.

And on the contrary, certain considerations are made in favor of *"non-specific plaque hypothesis"*, where most of oral diseases are generated by common *non-specific* deteriorating activities of total dental microbial mass despite their species origin.

To date the first hypothesis has got more confirmations indicating the prevalence of definite bacterial representatives in carious or periodontal lesions. Nonetheless, violation of oral hygienic measures results in rapid growth of bulk microbial biomass thereby elevating the risk of emergence of dental pathology. All this emphasizes the deleterious role of any shift in balance of normal oral microbiota.

But further progression of oral diseases inevitably leads to selection of the limited number of dental and periodontal pathogenic species responsible for basic disease course.

All infections of oral cavity are divided according to affected anatomical region.

Among them are ***dental*** (*caries* and *pulpitis*), ***periodontal*** (all forms of periodontitis) and ***gum diseases*** (*gingivitis*); infections of oral mucosa (*stomatitis*) and salivary glands; suppurative infections of neck and orofacial area (facial bones periostitis and osteomyelitis, sinusitis, lymphadenitis, soft tissue infections of neck and face). These primary infections may spread from initial sites resulting in ***odontogenic*** life-threatening regional or systemic disorders and complications (retropharyngeal, mediastinal or intracranial abscesses or phlegmons; and in exceptional cases, sepsis and septic shock).

Besides mentioned above, in some cases various ***non-odontogenic*** diseases of neck and facial area can arise. They comprise a number of purulent infections – folliculitis, furuncles (boils) and carbuncles, lymphadenitis, erysipelas, secondary hematogenous osteomyelitis and others.

And finally, orofacial area is commonly affected in patients suffering from the variety of ***specific infectious diseases*** (actinomycosis, tuberculosis, syphilis, diphtheria and many others).

Pathogenesis of Oral Infections: Common Steps

Infection-associated pathology of oral cavity commences from ***microbial adherence*** to dental and/or mucosal tissues resulting in *dental plaque formation* (microbial *biofilm*). Tight attachment of bacteria to host cells ensues from selective binding of multiple microbial *adhesins* to *membrane cellular receptors* of variable specificity.

Numerous groups of ***bacterial adhesins*** comprise cell wall polysaccharides, teichoic acids, certain bacterial enzymes, e.g., glycosyltransferases, polysaccharide-binding proteins or *lectins* and many others. Their counterparts – receptors of cell membranes – belong to exuberant groups of surface molecules such as mucins, lectins, integrins, members of immunoglobulin superfamily receptors, prolin-containing proteins, various glycoproteins, antibodies together with the considerable amounts of membrane-absorbed bacterial components (glycosyltransferases, glucans, etc.) In substantial number of cases this firm binding demonstrates moderate or low specificity. For instance, bacterial lipoteichoic acids bind to all negatively charged membranes by means of calcium ions.

Nevertheless, selective ***colonization*** of bacteria upon oral epithelium is promoted by specific interactions of microbial pathogens with host cells. In fact, *Actinomyces naeslundii* binds to superficial cell antigens by I^{st} type fimbria; *S. mutans* reacts with host prolin-enriched proteins, *S. gordonii* – with oral amylase, *S. sanguis* interacts with sialyl-containing oligosaccharides of MG2 mucins.

Moreover, the bacteria dramatically enhance primary oral colonization making tight cross-linkages between attached microbial cells.

The process of bacterial cells cross-binding is termed as ***coaggregation***. The most common is *intergeneric coaggregation* that

involves bacterial species from various genera. *Intrageneric coaggregation* occurs more seldom albeit it is typical for oral viridance streptococci. In latter case coaggregation is mediated by lectin binding.

Bacterial coaggregation entails the formation of microbial biofilm and dental plaque.

Synthesis of exopolysaccharides (glucans) from sucrose- or glucose-containing nutrients by *Streptococcus mutans* plays pivotal role in coaggregation of dental pathogens. Streptococcal glycosyltransferases produce polymeric glucans attached to microbial envelope. This elicits tight binding of streptococci to dental surface as well as to other bacterial cells via numerous microbial lectins.

Furthermore, the oral pathogens can directly impair host immune response making havoc of host defense factors. For instance, a great number of pathogenic bacterial species (e.g., *S. sanguis, S. oralis, S. mitis, Porphyromonas gingivalis, Prevotella intermedia, Capnocytophaga spp.* and others) are capable of producing high-rate IgA-proteases that actively destroy salivary sIgAs. This substantially lowers the grade of oral cavity protection.

Overall, under poor situation with oral hygiene, malnutrition or starvation conditions, or host endocrine and immune disorders bacterial colonization of oral cavity progresses rapidly. It biases the established equilibrium among normal oral microbiota towards the prevalence of pathogenic bacterial species. This in turn leads to preponderance of aggressive influences over protective ones within oral cavity. Finally the defense barriers can be broken down, and deleterious activities of microbial and non-microbial origin simulate the progression of certain oral disorders – e.g., dental caries, pulpitis or periodontitis.

Dental Caries

Caries is *dental disease of bacterial origin* affecting all of *dental hard tissues* (enamel, dentin and cementum) that is followed by demineralization and progressive decay of tooth structure *resulting in cavity formation*.

For about 2.5 billion people (approximately one third of global population) demonstrate dental caries of permanent teeth.

Usually caries emerges as local bacterial process. It starts from dental plaque expansion. Common tooth sites affected by caries are coronal surfaces, especially their fissures and pits. The disease can arise also on bare parts of dental roots in case of gingival recession.

General scheme of *pathogenesis of dental caries* looks as follows: demineralization (primarily, decalcification) of tooth hard tissues is caused by accumulation of organic acids in the offing of dental surface.

The rise of concentration of organic acids in oral cavity ensues from fermentation of food-derived carbohydrates (e.g., sucrose) by certain ***acidogenic species*** of indigenous oral microflora. The shift of pH towards acidity creates the conditions for selective propagation of so-called ***aciduric bacteria*** or capable of tolerating acids. These bacteria intensify acid production.

Organic acids (lactate and others) dissolve dental tissues resulting in tooth decalcification. A crucial pH level for start of tooth demineralization should be equal or less than 5.0-5.5.

A vast number of experiments and clinical observations confirmed the major role of viridance streptococci (and first of all – of species ***S. mutans*** and ***S. sobrinus***) in caries emergence and progression. Acidification of microenvironment spurs the next growth of lactobacilli in primary lesions.

These two bacterial groups can rapidly metabolize carbohydrates predominantly with lactic acid byproducts. First steps of the disease are related with *S. mutans* and *S. sobrinus* activity. Lactobacilli grow more slowly being concurrent with clinical signs of caries. Thus ***streptococci are cariogenic bacteria*** that initiate caries of dental tissues whereas lactobacilli are more responsible for disease progression.

Pathogenicity of *S. mutans* is maintained by its high adhesive capacity to dental enamel that stimulates dental plaque growth. *S. mutans* produces the enzymes glycosyltransferase and fructosyltransferase. They polymerize food-derived glucose and fructose into insoluble polysaccharides glucan and fructan. Glucan is the leading substance promoting adherence and coaggregation of *S. mutans* and other bacteria with reinforcement of dental plaque structure. For example, actinomycetes reside in dental biofilm via binding with their fimbria to biofilm glucans.

At neutral pH dental plaque harbors relatively low amounts of *S. mutans* and lactobacilli. By contrast, abundant consumption of sugar-containing nutrients results in their active microbial fermentation yielding lactic acid as the main byproduct that lowers dental plaque pH. This in turn dampens the growth of many resident bacterial species, such as *S. mitis, S. oralis, S. sanguis*, but accelerates propagation of *S. mutans* and lactobacilli. It biases local tooth metabolism to progressive demineralization. If oral defense factors (mainly, salivary flow and bicarbonate buffer) are unable to neutralize detrimental activity of pathogenic microflora the dental caries ultimately appears.

Meanwhile, the list of cariogenic microbial pathogens is not limited with the above mentioned bacterial species. In parallel with streptococci and lactobacilli the carious lesion confines broad spectrum of indigenous dental microflora. It was established quiet recently by methods of molecular genetic analysis (polymerase chain reaction or PCR, ribotyping, DNA and RNA microarray hybridization analysis) that many other bacterial species tamper with dental caries

Among them are *Actinomyces gerencseriae*, *Bifidobacterium spp.*, *S. salivarius*, *S. constellatus*, *S. parasanguinis* and others. In particular, *Actinomyces gerencseriae* is supposed to play a role in caries initiation, whereas the activity of bifidobacteria accounts for profound caries. On the contrary, domination of *S. sanguis* in dental plaque slows down the disease progression. Thus, dental caries results from deranging of complex multiple interplays normally established within oral microbiota.

If arisen but not treated, ***dental caries*** passes through several consecutive ***steps***:

(1) initial caries;
(2) superficial caries;
(3) moderate caries;
(4) deep caries
(5) deep complicated caries.

Initial caries appears as primarily white, then yellowish and later brownish spot. It is characterized by local tooth demineralization without visible structural changes. Initial caries is reversible if active mouth hygiene and fluoridation will be done.

Superficial caries affects enamel demonstrating wedge-shaped enamel defects but without dentin involvement.

Moderate caries corresponds to marked dentin damage.

Deep caries is followed by deep dentin penetration in closest vicinity to the pulp.

Deep complicated caries results in opening of the pulp cavity with pulpitis, periodontitis or abscess formation.

This division is generally consistent with *WHO classification* that includes four grade scale:

D1. clinically detectable enamel lesions with intact (non-cavitated) surfaces;
D2. clinically detectable cavities limited to enamel;
D3. clinically detectable cavities in dentin;
D4. lesions extending into the pulp.

Treatment and Prophylaxis of Caries

The treatment of dental caries depends on its stage. Initial caries and non-cavitated lesions don't need operative treatment. As the initial caries is reversible, enhanced oral hygiene favors remineralization. Topical fluoride therapy, e.g., fluoride varnish, demonstrates high preventive and treatment efficacy.

Cavitation requires restorative dentistry with operative interventions. All of the destroyed tissues should be removed with subsequent cavity filling.

Caries prophylaxis is primarily based on adequate oral hygiene and proper dietary recommendations with limited consumption of food sugars (e.g., "table sugar") and sticky foods like candies. A proper dental hygiene presumes regular teeth cleaning with toothbrushes and interdental brushes, flossing, the use of chewing gums with xylitol, etc.

Fluoride- and biocide-containing toothpastes evidently foster caries prophylaxis: oral antiseptics inhibit the growth of cariogenic microflora; fluorides stimulate calcification of dental hard tissues. Usage of dental sealants isolate teeth surface from aggressive external influences.

Specific prophylaxis of caries by vaccination still remains the subject of experimental medical design. Based on genetically modified strains of *S. mutans* or lactobacilli several experimental anti-caries vaccines undergo clinical trials now but their preventive efficacy requires further unbiased confirmation.

The results of caries prevention by topical applications of soluble antigens derived from cariogenic bacteria also remain controversial and need further elucidation.

Pulpitis

Pulpitis or ***inflammation of dental pulp*** arises predominantly as *complication of deep caries*, where profound dentin decay provokes pulp exposure to aggressive activity of microbial and other inflammatory factors.

Emergence of pulpitis is stimulated also by dental traumas, chemical irritation of pulp with dental restorative materials (e.g., sodium fluoride or phosphoric acid), surgical treatment of periodontal diseases or other medical interventions.

Nonetheless, it is obvious that the major role in etiology of pulpitis should be reserved for infectious agents. A multitude of oral pathogens may participate in pulpitis. Most common causative agents are numerous species of ***α-hemolytic streptococci***, representatives of ***gram-negative non-sporeforming anaerobic rods*** (bacteroids, fusobacteria, porphyromonads, prevotellas) and ***gram-positive anaerobic cocci*** (or ***GPAC***) – peptococci and peptostreptococci; actinomycetes and lactobacilli.

From carious cavity the bacteria enter the primarily sterile pulp through dentinal canaliculi, in some cases – by apical channel of dental roots. Also pulpitis might be borne from extradental infectious sites, such as infected gingival pockets, or as the result of sinusitis or orofacial osteomyelitis. The spread of hematogenous infection into the pulp is seldom observed.

Acute pulpitis is characterized by sudden onset and rapidly progressive inflammation with edema and sharp pain. It impairs dental blood supply.

Reactive inflammatory response in pulp is promoted by activity of innate immune cells against microbial pathogens. They comprise neutrophils, dendritic cells, T cells, natural killer cells, macrophages, odontoblasts. All these cells produce exuberant amounts of antimicrobial peptides, cytokines, chemokines and enzymes.

In several hours active purulent exudation may lead to periodontal inflammatory infiltrations or abscesses. If not treated, acute pulpitis exerts pulp necrosis, sometimes complicated with apical periodontitis; in case of modest activity it resolves into chronic process.

The treatment of reversible pulpitis foremost implies entire and high-quality treatment of caries. Removal of hard tissue decay and cavity restoration dampens inflammation and causes pulp healing.

Irreversible pulpitis with non-vital pulp requires endodontical treatment followed by removal of irreversibly damaged pulp.

Antimicrobial therapy is applied in cases of infection spread from pulp into surrounding tissues resulting in periodontitis, periostitis, regional lymphadenitis, or other complications. Beta-lactam antibiotics, doxycycline or anti-anaerobic drugs (metronidazole, clindamycin) can be administered.

Prophylaxis of pulpitis is non-specific. It depends on adequate dental care.

Periodontal Diseases

Periodontal pathology is the group of inflammatory diseases of infectious origin affecting any of tooth-supporting tissues (alveolar bone, periodontal ligament, cementum and gingiva).

Periodontal diseases are induced by deleterious activity of infectious agents concentrated in dental plaque. Poorly manifested, microbial pathogens stimulate local inflammatory responses that eventually lead to tissue atrophy with progressive collagen loss from tooth-supporting structures.

Inflammatory periodontal disorders are divided into 2 main categories: ***gingivites*** and ***periodontites***.

More than 50% of adult population have gingivitis and above 30% suffer from periodontitis.

Gingivitis

Gingivitis is the inflammatory gum disease. It is characterized by superficial inflammation affecting gums only. In these cases dental hard tissues and dental ligament still remain intact; and the depth of periodontal pockets doesn't exceed 3 mm.

Gingivitis begins from dental plaques expansion over gingival margin. Normally, progression of dental plaque is strictly limited by adequate dental hygiene that removes the most of oral pathogens. As the result, only low amounts of facultatively anaerobic gram-positive bacteria remain within gingival crevice. But in case of gingival inflammation total number of microbial cells increases rapidly up to 10-20 times from initial. It is followed by active preponderance of anaerobic gram-negative bacterial species amongst crevicular microbiota.

Non-specific (plaque-induced) *gingivitis* is the disease of evident polymicrobial nature. *S. sanguis, S. mitis, Fusobacterium nucleatum, Prevotella intermedia, Actinomyces naeslundii genospecies 2* (formerly known as A. viscosus), bacterial members from genera *Veillonella, Wolinella, Capnocytophaga* are commonly isolated.

By contrast, non-plaque-accociated gingival lesions comprise *specific bacterial, fungal and viral gingivites*, caused by certain microbial pathogens. These infectious agents are able to exert the direct damage of gum and oral mucosa in the course of primary infection.

Non-infectious secondary gingivites may follow systemic autoimmune or genetic disorders; or traumatic lesions.

Beyond the vast number of non-specific (or plaque-induced) microbial gingivites there is a special form of bacterial disease known as ***acute necrotizing ulcerative gingivitis*** or ***ANUG***.

It affects predominantly young persons, demonstrating sharp onset and severe pain due to *necrosis of interdental papilla* (gingival parts between the teeth). This acute gingival damage was primarily described by French physician H. Vincent and thereafter named as "Vincent's angina" or "Vincent's disease".

During the years of World War I the disorder was known as "trench mouth", afflicting predominantly military staff. However, it may occur in any person in conditions of starvation or malnutrition and under stress.

H. Vincent ascribed the etiology of the disease to mixed spirochetal and fusobacterial infection mainly due to permanent detection of this agents within specific oral lesions. The presence of large spirochetes with irregular coils carrying more than 20 fibrils was commonly found in clinical specimens taken from these patients.

Now it is generally ascertained that acute necrotizing ulcerative gingivitis arises from complex polymicrobial infection, where major role belongs to oral spirochetes and the members of gram-negative anaerobic *Prevotella intermedia* species.

The ***treatment of microbial gingivitis*** includes the administration of oxidizing agents (hydrogen peroxide or iodine) and the use of antimicrobial drugs, affecting anaerobic bacteria, such as metronidazole. Rinsing of oral cavity with solutions of oxidants (e.g., hydrogen peroxide) prevents the emergence of acute necrotizing ulcerative gingivitis. Overall, adequate prophylaxis and treatment ensures favorable prognosis of these disorders.

Periodontitis

Periodontitis is the inflammatory polymicrobial infectious disease affecting supportive dental tissues that if not treated, leads to tissue attrition with progressive collagen degradation, alveolar bone resorption and eventual tooth destabilization or loss.

Pathogenesis of Periodontitis

Pathogenesis of periodontal diseases is a complex multifactorial process comprising dental plaque overgrowth, exuberant accumulation of microbial wastes and virulence factors ultimately resulting in local progressive inflammatory response.

In the course of disease the gingival crevice deepens over 3 mm and transfigures into periodontal pocket gradually expanding from 4 to 10-12 mm and even more. Every pocket may contain 10^7-10^9 of microbial cells. Detrimental activity of microbial pathogens harbored in the pocket accounts for disease progression and tissue destruction.

The composition of local microbial communities changes grossly following the development of periodontitis.

As early as in 1998 S. Socransky with coworkers proposed to divide the members of oral microbiota into *separate groups* that *correspond to healthy or pathological conditions* found within oral cavity.

Each group harbors a number of related microbial species that are commonly isolated at certain steps of dental plaque growth or, by contrast, when pathology arises. However, there are striking dissimilarities observed between the groups. Therefore, every group reflect unique colonization pattern essential for various microbial communities.

By S. Socransky, different "***colors***" were assigned to these microbial clusters, named as "***complexes***".

It was pointed out that "*purple*", "*cyan*" "*yellow*" and "*green*" complexes comprise early first colonizers of the tooth surface especially of its subgingival sulcus. Thus, the members of these complexes were primarily ascertained as the residents of healthy gums.

"***Purple*** or *magenta* ***complex***" is closely associated with healthy gingival state and includes species *Veillonella parvula* and *Actinomyces odontolyticus*.

"***Yellow*** complex" encompasses a number of streptococci (*S. sanguis, S. mitis, S. gordonii* and *S. intermedius*), "*cyan* complex" – numerous actinomycetes.

The bacteria from purple and yellow complexes are regarded as *protective microbial agents* demonstrating antagonistic activities against pathogenic microflora.

"***Green*** complex" was found to contain diverse microbial agents such as *Eikenella corrodens, Aggregatibacter actinomycetemcomitans serotype a, Campylobacter concisus, Capnocytophaga spp*. It has been shown after close scrutiny that species of green complex can actively

participate in progression of serious dental pathology, e.g., periodontites with tissue destruction.

Finally, the bacteria from *red* and *orange complexes* demonstrate intimate association with oral pathological conditions.

Three members of ***red complex*** – gram-negative obligate anaerobes *Porphyromonas gingivalis, Tannerella forsythia* (former *Bacteroides forsythus*), and *Treponema denticola* are the pivotal periodontal pathogens commonly isolated in ***chronic periodontitis*** with deep pockets and gingival recession.

The ***orange complex*** embraces the variety of anaerobic pathogens *Prevotella intermedia* and *Prevotella nigrescens*; *Streptococcus constellatus, Eubacterium nodatum, Peptostreptococcus micros*, several species from genera *Fusobacterium* (*F. nucleatum, F. periodonticum*), and *Campylobacter* (e.g., *C. rectus*).

The bacteria of ***orange*** complex are associated with ***gingivitis*** and gingival bleeding. They are tightly related with red complex members demonstrating mutual pathogenesis.

Three other pathogenic species, namely *A. actinomycetemcomitans serotype b, Selenomonas noxia* and *Actinomyces naeslundii genospecies 2* (formerly *A. viscosus*) don't pertain to any outlined complex but intensively impact on progression of dental pathology as well.

Division of bacteria into pathogenicity groups or "complexes" strongly correlates with clinical situation in periodontal diseases. These disorders are proven to be ***inflammatory*** injuries of ***polymicrobial origin***.

Normal microbiota of subgingival plaque commonly harbors facultatively anaerobic gram-positive bacteria (mainly, streptococci), actinomycetes and anaerobic gram-negative veillonellas (*purple* and *yellow complexes*), but only 5% of spirochetes or anaerobic motile rods.

In case of irregular and poor oral hygiene the expansion of dental plaque accelerates secretion of crevicular fluid and stimulates local inflammatory response. If not recovered, microbial metabolism and inflammation turns down crevicular redox potential thereby affording the growth of anaerobic bacteria. Most of them release powerful virulence factors with proinflammatory (e.g., bacterial LPS), enzymatic (collagenase, elastase, hyaluronidase, etc.) and toxic activities. gram-negative anaerobes from genera *Porphyromonas, Prevotella, Fusobacterium, Tannerella, Aggregatibacter, Capnocytophaga, Wolinella*, and *Treponema* substantially worsen the local periodontal status.

Tissue matrix metalloproteinases and bacterial hydrolytic enzymes destroy supportive dental surroundings with marked collagen degradation.

Persistent inflammation converts into chronic periodontal disease. The latter results in dental pocket excavation, gingival recession and final tooth destabilization.

Overall, in course of chronic periodontitis naturally present bacteria of "purple" and "yellow" complexes are gradually substituted by periodontal pathogens of "red" and "orange" microbial groups. In these conditions gram-negative bacteria comprise 75% of total cells, and what's more, 90% pertain to strict anaerobes.

Clinical Variations of Periodontitis

There are several kinds of periodontitis that are different in clinical course.

Among them are ***chronic*** *periodontitis*, ***aggressive*** *periodontitis*, periodontitis as a manifestation of systemic diseases; periodontites, associated with genetic or hematological disorders. All of these forms can be *localized*, *generalized* or *refractory* (see Table 8).

Table 8
Classification of periodontal diseases
from The American Academy of Periodontology, 1999

I. Chronic periodontitis	**II. Aggressive periodontitis**	**III. Periodontitis as a manifestation of systemic disease**	**IV. Necrotizing periodontal disease**
Localized *Generalized* *Refractory*	Localized Generalized Refractory	Associated with hematological disorders Associated with genetic disorders	

Also periodontites are divided into the disorders affecting individuals of 35 years or younger, and periodontites of adults (the disease in persons over 35).

At last, there are special clinical forms of periodontitis, for instance, *local juvenile periodontitis* and necrotizing periodontal disease.

Local Juvenile Periodontitis

Local juvenile periodontitis predominantly affects teenagers with annual morbidity rate from 1 to 5 in 1,000 of population.

In absence of treatment, rapid degenerative lesions after the disease emergence lead to ultimate dental loss.

In spite of its severity, the low volume of dental plaque is characteristic for this pathology. It damages mainly molar and incisor teeth without extensive plaque or calculus formation.

Unlike other kinds of periodontites, the disease has narrow range of microbial species in its etiology. In most of cases microaerophilic bacteria ***Aggregatibacter actinomycetemcomitans*** are isolated from periodontal lesions. They are cultured on malachite green-bacitracin selective medium.

This periodontal pathogen produces potent ***virulence factors***, such as ***LPS*** with proinflammatory activity and highly active ***leukotoxin***.

Leukotoxin suppresses the actions of neutrophils thus ensuring microbial penetration into nethermost tissues. In some cases the infection may spread further to the bloodstream. Generalization of infectious process triggers both local and systemic immune responses.

Manifested severe forms of the disease are supposed to evolve in children, which carry genetic defects in neutrophil chemotaxis. Protracted course of local juvenile periodontitis allows other microbial species (e.g., anaerobic bacteria) to participate in disease pathogenesis thereby worsening its prognosis. Nevertheless, administration of broad spectrum antibiotics (doxycycline or metronidazole) successfully interrupts the infectious process.

Periodontitis of Young Individuals (Early-onset Periodontitis, Aggressive Periodontitis)

This form of the disease afflicts the young persons below the age of 35. It may affect from 0.5 to 2% or even more of children and young people. The most of disease cases are followed by dental plaque broadening and calculus formation. The infection moves to periodontal tissues and actively propagates there. In case of aggressive disease course eventual dental loss is possible even before the age of 20.

It was long-time supposed that a major role in etiology of early-onset aggressive periodontitis belongs to periodontal pathogen *A. actinomycetemcomitans.* However, it has been found by now that the

disease emergence and progression is not primarily related with microaerophilic bacteria but depends on limited number of obligate anaerobic bacterial species *Treponema denticola*, *Prevotella intermedia*, *Tannerella forsythia*, *Porphyromonas gingivalis* and some others.

The prophylaxis and treatment of this ailment is generally similar to above-mentioned clinical forms of the disease.

Adult Periodontitis (or Chronic Periodontitis)

It is one of the most common infectious diseases affecting adult population. Usually it demonstrates slowly progressive course with multiple dim exacerbations. It is characterized by low-grade or moderate inflammation resulting in degenerative lesions of periodontal tissues and tissue atrophy.

The disease is evidently of ***polymicrobial origin*** with high ***prevalence of anaerobic bacteria***. The culturability of most of this species is very low; that's why they can be discovered only by methods of molecular genetic analysis (polymerase chain reaction or PCR and DNA hybridization).

By lowering of redox potential in periodontal pocket, a selective growth of bacteria from red and orange complexes accompanied by numerous microaerophilic pathogens is stimulated. The association between microaerophil species *A actinomycetemcomitans, Wolinella recta, Eikenella corrodens* and obligate anaerobes *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Prevotella intermedia*, *Fusobacterium nucleatum* and some others is typically observed.

Peri-implantitis, the disease resembling periodontitis, but affecting dental implant surroundings, demonstrate similar behavior. Failing implants and residual teeth can be the subject of bacterial attack.

Despite the striking variability of microbial communities residing in dental pockets, the therapy of chronic periodontites should be oriented on prevention or restrain of only limited number of the most active periodontal pathogens. As they are predominantly anaerobic, the administered drugs must impact mainly this group of bacteria. Most effective are metronidazole, clindamycin, doxycycline or beta-lactams. They can be used locally by applications of polymeric antibiotic-containing films into dental pockets or by systemic administration in severe cases.

Drug therapy should be only the constitutive part of complex versatile treatment of chronic periodontitis. It comprises also the debridement

procedures (scaling and root planning) and, if needs, dental surgery. Taken together, these procedures terminate the disease progression and prevent tooth loss.

Periostitis and Osteomyelitis of Mandibular and Maxillofacial Region

Jaw ***periostitis*** is the inflammation of periosteum of corresponding bone.

In majority of cases it ensues from complicated acute periodontitis or as the result of exacerbation of chronic periodontal disease. More seldom it arises from periapical abscess, suppurative radical or follicular cysts, wounds after dental extraction, etc.

Osteomyelitis is the inflammatory process affecting bones and/or bone marrow.

Osteomyelites are divided into *suppurative* or infectious (the most common) and *non-suppurative* (e.g., after aseptic traumatic injury). They are also classified into *acute* and *chronic* (with duration more than 1 month).

In dentistry osteomyelites of orofacial area can be odontogenic (from dental infection) and non-odontogenic (hematogenous, post-traumatic and others).

The most commonly isolated microbial pathogens that cause periostitis and osteomyelitis are ***S. aureus*** (more than 80% of cases in adults), group A streptococci, *Enterobacter* species; and *H. influenzae* in children.

Suppurative post-traumatic osteomyelitis can be provoked by *S. aureus*, *Enterobacteriaceae* members or *P. aeruginosa*.

Complex treatment of periostitis and osteomyelitis encompasses adequate surgery of infectious sites and antimicrobial treatment with antiseptics and antibiotics. Antiseptics (e.g., *chlorhexidine*) are applied locally; antibiotics should be administered according to the results of laboratory testing of microbial antibiotic resistance. The most commonly used drugs for antimicrobial treatment in these cases are beta-lactams in combinations with β-lactamase inhibitors, e.g., *amoxicillin-clavulanic acid*; in case of *P. aeruginosa* infections carbapenems, piperacillin/tazobactam or ceftazidime are used.

Odontogenic Maxillary Sinusitis

Acute or chronic ***sinusitis*** is relatively rarely caused by tooth infection.

Nevertheless, odontogenic maxillary sinusitis makes up to 20% of total cases of maxillary sinusitis.

The most common agents of odontogenic maxillary sinusitis are ***S. pneumoniae***, ***H. influenzae***, or *M. catarrhalis*, sometimes *S. aureus* or non-sporeforming anaerobic bacteria. Vaccination against *H. influenza* type B (or Hib) substantially reduced the incidence of sinusitis caused by this bacterial pathogen.

In most cases of acute sinusitis antibiotic treatment is not used. If the disease exceeds 10 days of duration, a short 3-7 days course of amoxicillin/clavulanate is recommended.

Suppurative Infections of Soft Tissues of Facial and Neck Areas

Suppurative odontogenic infection of face and neck soft tissues is rare but highly severe complication of primary infections of oral cavity. It affects muscles, subcutaneous fat, blood vessels, fibrous connective tissue and fascia of orofacial and neck regions.

These injuries are clearly of polymicrobial nature with active participation of streptococci, staphylococci, bacteroids, fusobacteria, peptostreptocci, in case of hospital infections – *Pseudomonas aeruginosa*.

Once appeared, suppurative infections spread from initial site resulting in *abscess* or *phlegmon* formation.

Tissue ***abscess*** is the localized cavity filled with the pus, bacteria, phagocytes and elements of destroyed tissues (or debris). It is surrounded with *abscess wall* or *capsule* made of inflammatory granulation tissue. Encapsulation acts to hold pus inside and prevents its further spread but the same time it restrains the activity of immune cells.

Phlegmon is the severe purulent inflammation of soft tissues that actively spreads from primary site without tendency to self-limitation. It affects muscles, fascias and adipose tissue resulting in non-capsulated pus accumulation amid muscles and fascial compartments.

Odontogenic phlegmons or abscesses originate from initial infectious sites located in teeth or periodontal lesions, inflamed salivary glands (*sialadenitis*), tonsil crypts, lymph nodes or other structures.

Depending on microbial virulence and immune reactivity, the course of infections might be drastic and aggressive with high fever and intoxication or, by contrast, torpid and faint. Anyway, both situations can't lead to self-recovery.

This pathology requires ***active surgical treatment*** and ***antibacterial therapy***. Patients should be treated with antibiotics according to estimated microbial drug resistance. Beta-lactams in combination with β-lactamase inhibitors, (e.g., *amoxicillin-clavulanate*) can be used as the drugs of first line.

Facial and Neck Lymphadenitis

Regional lymphadenitis of neck and facial area usually follows the inflammatory infections of oropharyngeal and facial zones.

Overall, sub-mandibular and neck lymphadenites can arise from *odontogenic* or *non-odontogenic* infections.

Sub-mandibular lymphadenitis and lymphadenitis of anterior and posterior neck surface in children before 4-5 are related mainly with viral infection.

Most common are so-called "***non-specific***" lymphadenites that pose the background for virtually any suppurative lesion of orofacial region.

"***Specific***" lymphadenites may arise after specific microbial antigen exposure, e.g., BCG vaccination against tuberculosis.

Abscesses of lymph nodes are provoked by secondary bacterial infection spread throughout regional lymphatic system. They are usually caused by typical number of infectious agents, such as streptococci, staphylococci, actinomycetes, gram-negative anaerobic microflora.

Antimicrobial treatment of lymphadenitis is performed as auxiliary measure in complex cure for primary oral infection of bacterial origin.

Odontogenic Bronchial and Pulmonary Infections

In certain cases dental and periodontal infections can elicit bronchial or pulmonary pathology.

As the oral cavity harbors myriads of microorganisms, some of opportunistic pathogens may cause respiratory diseases (bronchitis or pneumonia) if they reach bronchial or pulmonary tree. Among them are pneumococci, klebsiellas, *S. pyogenes*, mycoplasmas, chlamydiae,

bacteroids, several uncommon bacterial agents such as *Moraxella spp., Kingella kingee, Acinetobacter baumanii,* or viruses (e.g., herpes virus).

Secondary bacterial pneumonias arise in patients under severe conditions affecting respiratory tract. They occur in case of aspiration of oral or gastric contents in patients with bedridden status or long-time unconscious; in postoperative patients, patients receiving mechanical ventilation, patients with debilitating neurologic disorders; immunocompromised individuals with primary or secondary immune deficiencies, etc.

Secondary pneumonias usually demonstrate *mixed polymicrobial infection*. They are caused by long list of opportunistic bacterial agents (e.g., staphylococci, enterococci, klebsiellas, pseudomonads, enterobacterial pathogens, anaerobic bacteria). In many situations this microflora can be of odontogenic origin.

Aspiration pneumonia occurs after inspiration of foreign substances that enter respiratory tract from outside (e.g., from oral cavity). Aspirates may contain oral secretions, acidic gastric contents, vomiting masses, foods, and multiple microorganisms. It starts as chemical and mechanical pneumonitis followed by progressive bacterial inflammation. Aspiration pneumonia often leads to lung abscess or pleurisy with empyema.

In most of cases this disease emerges as *hospital acquired pneumonia*. It is caused predominantly by gram-negative anaerobes, *S. aureus, Klebsiella pneumoniae* or *Pseudomonas aeruginosa.*

Ventilator-associated pneumonia or ***VAP*** is one more type of *hospital-acquired* (or ***nosocomial***) *pneumonia*. It may develop in patients undergoing mechanical ventilation within intensive care units.

Severe *hospital pathogens* demonstrating *striking resistance to antimicrobial therapy* are commonly isolated in case of VAP. Among them are methicillin resistant *Staphylococcus aureus* (or ***MRSA***), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, and *Serratia marcescens*.

Laboratory diagnosis of nosocomial pneumonias includes specimen sampling followed by identification of isolated bacterial cultures and their antibiotic susceptibility testing.

Patient's sputum, airway samplings, endotracheal aspirates, bronchoalveolar lavage fluids, pleural fluid cultures are usually examined. Direct identification of bacterial strains in clinical specimens is performed by polymerase chain reaction (PCR).

The treatment of severe pneumonias caused by multi-resistant hospital strains requires *high-dosage combined antibiotic treatment* with broad-spectrum antibiotics.

Antipseudomonal cephalosporins (cefoperazone or ceftazidime), carbepenems, combined β-lactam/β-lactamase inhibitor-containing antibiotic piperacillin-tazobactam, fluoroquinolones (levofloxacin) or aminoglycosides (amikacin or tobramycin) are generally administered.

Gram-positive multidrug resistant microflora, e.g. methicillin resistant *Staphylococcus aureus*, is treated with linezolid or vancomycin.

Odontogenic Sepsis

Sepsis is a ***systemic*** bacterial or fungal disease followed by life-threatening ***organ dysfunction*** caused by a ***dysregulated host response*** to infection.

The term "sepsis" originates from Greek: "*putrefaction*" or "*rotten flesh*" indicating deep impairment of the whole body due to the severe infection.

Odontogenic sepsis is induced by ***infection residing in oral cavity***. It becomes much more likely if odontogenic infection spreads through the fascial spaces to the head and neck.

Primary focus of infection may localize in mandibular (about 70%) or maxillary (for about 30%) zones. If not restricted, the infection can spread further to unaffected anatomical regions and tissues along the sites of least resistance.

When arisen, the septic state develops into mortal systemic inflammatory disease via several consecutive phases. They reflect the levels of disease progression followed by growing incapacity of host defense to restore normal body functions.

The ***stages of systemic bacterial infection*** expand as follows: (1) bacteremia phase; (2) sepsis; (3) septic shock.

Bacteremia means the presence of ***viable*** bacteria in bloodstream. Nevertheless, bacterial dwelling in blood creates only necessary but not yet sufficient condition for sepsis development.

Bacteremia is generally divided into transient, intermittent, or continuous depending on duration of bacterial stay in blood.

Transient bacteremia lasts from a few minutes to few hours. It may occur under ordinary medical manipulations (i.e., dental extractions or percutaneous injections and catheterizations).

Intermittent bacteremia appears in case of presence of localized infectious focus such as abscess or osteomyelitis. It is characterized by periodical entry of bacteria into bloodstream with subsequent clearance.

Finally, ***continuous*** bacteremia occurs when the infection long-time resides directly within blood vessels or cardiac valves, i.e., in cases of infective endocarditis or vessel graft infections.

But the progression of conventional bacteremia into systemic disease or sepsis requires ***extra-conditions*** that must aggravate primary course of infection.

The bacteria should demonstrate high virulence with powerful adhesive, invasive and immunosuppressive capacity; on the other hand, host defensive systems exhibit ***ineffective*** inflammatory response or, conversely, fall into anergy both unable to clean the invaded pathogen.

Since early 1990s sepsis was regarded as ***infectious*** *systemic inflammatory response syndrome* (or ***SIRS***) that leads to severe tissue damage and organ dysfunction. Every SIRS case shows characteristic clinical manifestations resulting in changes of body temperature (e.g., <36°C or >38°C), pulse and respiratory rates, and white blood cell count.

However, it became evident that the term "SIRS" has more expanded meaning than sepsis itself. According to the above criteria, SIRS as systemic inflammation can arise from both infectious and non-infectious insults. For instance, non-infectious SIRS emerges in course of pancreatitis, burn disease, multiple trauma with massive tissue injury, or hemorrhagic shock.

Moreover, intensive systemic immune response against the infection resembles clinical conditions of SIRS, but it successfully eliminates the invaded microbial pathogen despite the development of active body inflammation.

As the result, in 2016 the concept of sepsis was revised by international Task Force that established International Consensus Definitions for Sepsis and Septic Shock.

According to current Definitions, ***sepsis*** is defined as "***life-threatening organ dysfunction*** caused by a ***dysregulated host response to infection***".

The infection might be *documented* or *clinically suspected*.

Pathogenesis of sepsis is based on abnormal hyper- or hypoactivity of immune response against systemic infection coupled with enhanced virulence of microbial pathogens.

Immune hyperactivity is followed by massive secretion of proinflammatory cytokines ("*cytokine storm*") from granulocytes, dendritic cells, macrophages and all other cells of innate response. This leads to

endothelial damage, deep microcirculation disturbances and intensive procoagulant activity that ensure inner organs malfunction.

And vice versa, highly virulent microbial pathogen causes similar body injuries on the background of inefficient immune response.

If not treated perfectly, ***sepsis*** progresses into ***septic shock*** with multiple organ failure and tissue damage.

Septic shock is defined as sepsis condition "in which ***particularly profound*** circulatory, cellular, and metabolic ***abnormalities*** are associated with a ***greater risk of mortality***".

Overall, sepsis mortality rate remains high even in developed countries. In Europe it exceeds 40% in cases of septic shock. Nowadays the disease is called as "hidden public health disaster". For instance, there are about 750,000 patients with sepsis registered in United States annually resulting in more than 200,000 death cases; and the total cost of treatment of hospitalized patients is equivalent to $20 billion.

Laboratory diagnosis of sepsis including its ***odontogenic*** forms poses serious difficulties.

The diagnosis must be established as soon as possible (in first few hours) while ***massive early and targeted antibiotic therapy*** substantially favors the prognosis. The presence of initial infectious focus (e.g., necrotizing soft tissue infection) should be confirmed or excluded within 12 hours.

However, repetitive blood bacterial cultures become positive in less than 50% of cases. Primary culturing should be done before initiation of antimicrobial treatment. The most commonly isolated microbial species (e.g., staphylococci, viridance streptococci, gram-negative anaerobes, enterococci, *Pseudomonas aeruginosa*) are identified and tested for antibiotic resistance according to standard protocols.

Current progress in laboratory diagnosis of sepsis is related with methods of ***molecular genetic analysis***. Multiplex real-time PCR tests and fluorescence in situ hybridization are used. Ideally they can detect individual microbial cells in 1 ml of blood.

Discovery of rapid reliable biomarkers of emergence of sepsis still remains the subject of intensive research. Laboratory monitoring of blood levels of biomarkers with relatively high specificity and sensitivity for sepsis (lactate, procalcitonin, C-reactive protein, IL-6 and some other cytokines) might be helpful in disease diagnosis and in control of treatment efficacy.

Treatment of odontogenic sepsis must be highly intensive and complex. It commences with complete surgical eradication of primary septic focus in orofacial or neck area.

Adequate and urgent antibiotic therapy that starts within 1 hour of sepsis diagnosis is the subject of ultimate clinical value. Initial empiric antibiotic treatment is performed before the results of antibiotic susceptibility testing and it can be changed after collecting of microbial resistance data.

High doses of beta-lactams combined with β-lactamase inhibitors, carbapenems, aminoglycosides (amikacin or tobramycin), fluoroquinolones or vancomycin for gram-positive microflora can be used. In case of fungal sepsis amphotericin B is administered.

The treatment is followed by intensive maintenance of body vital functions – fluid resuscitation, the control and maintenance of blood pressure, cardiovascular, respiratory and renal support.

Prophylaxis of odontogenic sepsis is essentially based on active elimination and treatment of primary infection focus located in oral cavity.

Bacteriological Testing in Dental Practice

Bacteriological analysis in patients with odontogenic diseases is performed in several clinical conditions:

a) in cases of ***suppurative infections*** of orofacial and neck areas; the main goal of this study is the isolation of causative agent and its antibiotic susceptibility testing;

b) in clinical cases of ***"specific" infections***, where oral cavity lesions arise in primary infectious diseases (e.g., diphtheria, syphilis, scarlet fever, tuberculosis and others); it fosters the establishment of correct etiological diagnosis of infection;

c) in cases of ***long-term oral lesions of unknown origin*** (unclear diagnosis).

Specimen taking for microbiology examination requires some common rules. Sampling should be done without any preliminary mouth treatment by antiseptics or other drugs and before tooth brush. Directly before taking oral cavity is rinsed with sterile warm saline. In case of oral ulcerative lesion its superficial covering film should be removed, and the specimen is delivered from lesion's bottom.

Without delay the material should be sent to the laboratory for culture examination. If proper nutrient media present, the inoculation can be done directly in dental cabinet.

Most common situations that demand bacteriological testing of odontogenic infections are related with cervical lymphadenitis, submandibular, retropharyngeal and other deep space infections, peritonsillar abscesses.

Specimen collection is made by aspiration or biopsy of inflammatory material from injured tissues and tissue spaces. Samples have to be placed into anaerobic transport container to make possible isolation of anaerobic bacteria, which are common in these conditions. Besides, facultatively anaerobic bacteria remain viable in anaerobic transport. All the specimens should be immediately delivered to the laboratory, where the isolation of microbial cultures and their antibiotic susceptibility testing are elaborated.

In odontogenic sepsis (as well as in other septic cases) the repeated blood sets taken from patient are examined. At least 2–4 blood culture sets are tested per septic episode.

For successful microbial recovery the most critical is the volume of blood that is collected by venipuncture. Not less than 20-30 ml of blood for every bottle with nutrient media should be taken. In adults 2-4 bottles for single blood culture set are used, at least one aerobic and one anaerobic. The amount of nutrient medium in every bottle has to be 200 ml or more to remit bactericidal activity of blood. Catheter-drawn blood cultures demonstrate a higher risk of external contamination.

In sepsis two and more blood culture sets must be taken sequentially in a short period of time before initiation of antibiotic treatment; after blood sampling empiric antibiotic therapy should be started immediately.

For ***rapid sepsis diagnosis*** nucleic acid amplification tests (*NAATs*) are used. Patients' plasma or serum is examined by multiplex real-time PCR or molecular hybridization tests.

Microbiological testing of saliva and gingival crevicular fluid ameliorates the diagnosis of periodontal diseases and helps to assess risks in patients with caries.

Saliva testing can be done both with resting (unstimulated) or stimulated salivary specimen.

Resting saliva is secreted predominantly by submandibular glands. It is collected by allowing a patient to expectorate saliva into a collection cup.

Stimulated saliva is derived mainly from the parotid gland; it can be taken by masticatory stimulus (e.g., a piece of wax). To get stimulated

salivary specimen, the patient chews a piece of wax for a 5 min expectorating saliva into a small receptacle at regular time periods.

Salivary samples are used for rapid chairside caries risk assessment tests. Risk assessment tests estimate total amount of the most cariogenic bacterial species, namely *S. mutans* and lactobacilli, by reaction of salivary aliquot with highly specific monoclonal antibodies against these pathogens.

Gingival crevicular fluid (GCF) is tested for laboratory diagnosis of periodontal diseases. GCF samples are obtained from periodontal lesions (e.g., gingival pockets) of the tested teeth. The teeth should be cleaned to remove any supragingival plaque, then isolated with cotton roll to prevent saliva contamination and dried with air. Standard endodontic paper points (e.g., of size 30) or collection paper stripes are inserted into the gingival crevice. Standardization is achieved by equal time of sampling.

Next step the obtained specimen is transferred to appropriate nutrient media for growth. Anaerobic culturing is preferable in this condition as the most active periodontal pathogens (i.e., *Prevotella intermedia*, *Tannerella forsythia* or *Porphyromonas gingivalis*) pertain to obligate anaerobic bacteria.

Swabs of dental plaques are used to evaluate the grades of common oral cleanliness. For example, the quantity of microbial ATP can be determined in this sample that correlates with total amount of bacteria present in dental biofilm.

MEDICAL VIROLOGY

Chapter 13
GENERAL CHARACTERISTICS OF VIRUSES

The History of Virus Discovery

*The beginning of the history of viruses has started from the discovery of tobacco mosaic disease causative agent, made by Russian scientist D.I. Ivanovsky in 1892. He has found this agent as the smallest size particle, capable of passing through bacterial filters, invisible in light microscope, and devoid of ability to grow in different nutrient media. Thus he described several major essential traits of viruses, albeit the term "***virus***" (Lat.* virus – poison*) appeared later. This name was proposed by the Dutch scientist M. Beijerinck in 1898. Finally it was applied to all of the similar infectious agents.*

General Characteristics of Viruses

All of the viruses demostrate the number of common properties:

*– molecular (***non-cellular***) structure;*

– viral genome represents only the **one type of nucleic acid** *– DNA or RNA that indicates* **DNA** *or* **RNA viruses***, respectively; viral nucleic acids can be organized as* **double-stranded** *or* **single-stranded***;*

– viruses are **obligate intracellular parasites** *capable of propagating solely in the living cells;*

– viruses are devoid of molecular structures for protein synthesis; the infected cell provides energy and ribosomal apparatus for successful viral propagation (synthesis of proteins and nucleic acids, viral assembly, etc.)

– viruses are the **minute** *microorganisms, ranging from about 15-20 nm to 400 nm in most of cases; viral particles are able to pass through the filter membranes, retaining the majority of bacterial cells.*

– viruses demonstrate **highly intensive genetic variability***; phylogenesis of viruses is governed by the laws of evolution;*

– viruses are **ubiquitous** *in nature; they can multiply in bacteria, algae, fungi, and protozoa as well as in plants and animals;*

– viruses **are not affected by antibiotics***; viral infections are treated by special class of antiviral drugs.*

The host range for a certain virus may be wide or extremely limited. Usually viruses can interact only with the cells of a few related species.

Classification of Viruses

Modern **classification of viruses** *is elaborated by* International Committee on Taxonomy of Viruses *(ICTV). ICTV is a committee of Virology Division of the International Union of Microbiological Societies.*

In contrast to any other biological objects, as ICTV states, "...nomenclature of viruses is independent of other biological nomenclature" and further "...virus taxon nomenclature is recognized as an exception in the proposed International Code of Bionomenclature".

The data concerning actual viral classsification are periodically published in ICTV Reports and releases. The last 9th Report was developed in 2009-2012; the latest release of virus taxonomy was presented in 2015.

According to ICTV principles, universal classification of viruses operates with taxonomic levels of order, family, subfamily, genus, and species.

Current version of international classification of viruses comprises 7 viral orders, 111 families that include at least 27 subfamilies and 609 genera with more than 3700 viral representatives. Nevertheless, hundreds of viruses are not classified yet.

Not all, but many viral **families** *are organized into* **orders**.

Virus order names are marked with the suffix **-virales**, *whereas the titles of viral families contain the suffix* **-viridae**.

For instance, several families are included into the order Mononega**virales***; among them* Filo**viridae**, Paramyxo**viridae** *and* Rhabdo**viridae** *harbor the large number of viruses, pathogenic for humans.*

Subfamilies have the suffix **-virinae**. *As an example, the family* Herpesviridae *comprises 3 subfamilies:* Alphaherpes**virinae**, Betaherpes**virinae** *and* Gammaherpes**virinae**.

Viral families and subfamilies encompass numerous genera. Genus names are ended with the suffix **-virus** *(e.g.* Orthomyxo**virus**, Hepato**virus**, Rubi**virus** *and many others.)*

Species *is the primary lowest taxonomic unit of international viral classification. Species name of virus may indicate virus locality, its susceptible host or virus-associated disease, e.g. measles virus.*

ICTV designates **viral species** *as "...a* **monophyletic** *group of viruses whose properties can be distinguished from those of other species by* **multiple criteria***". Among these multiple criteria are:*

– the degree of relatedness of viral genomes or genes;

– natural and experimental host range;
– cell and tissue tropism;
– viral pathogenicity;
– vector specificity;
– antigenicity, etc.

Monophyletic *group means the closely related population of viruses where all the members* **originated from the common ancestor**.

This is determined on the base of viral phylogenetic analysis *with* **molecular genetic methods**, *mainly by* **sequencing of viral nucleic acids** *(DNA or RNA).*

Nucleic acid sequencing and sequence comparison evaluates the genetic relatedness *and* origin *of members of viral species.*

In case of absence of full genomic data the sequencing of viral proteins is proposed.

However, the uniform genetic criteria suitable for delineation of any viral species are not elaborated. For instance, the plant viruses from the family Geminiviridae *are established from the same species in case of genetic identity from 90%, whereas for ebolaviruses this criterion is equal to 70% of their RNA relatedness.*

That's why above mentioned additional criteria for virus speciation are used.

Taxonomic ranks from genus and above are described by ICTV as the group of related taxa of lower level (e.g., for viral genus – the number of viral species) that share certain common properties.

Taking into account the existing difficulties of viral taxonomy accompanied with low accessibility of genetic sequencing and other advanced molecular tests for most of clinical laboratories parallel viral division into 7 distinct groups is commonly used in practice.

This classification was primarily proposed by Nobel Laureate US virologist D. Baltimore still in 1971.

It grounds on the basic structure of viral genome – type of genomic nucleic acid (DNA or RNA), number of nucleic acid strands (double-stranded or single-stranded), polarity of viral RNA – positive-sense or (+) RNA and negative-sense or (–) RNA; mode of replication of viral genome.

There are the next viral groups according to Baltimore classification:

group I – double-stranded DNA viruses;
group II – single-stranded DNA viruses;

group III – double-stranded RNA viruses;
group IV – single-stranded RNA viruses – positive-sense RNA;
group V – single-stranded RNA viruses – negative-sense RNA;
group VI – positive-sense single-stranded RNA viruses that replicate through a DNA intermediate
group VII – double-stranded DNA viruses that replicate through a single-stranded RNA intermediate

The groups VI and VII comprise the viruses with replication via the reverse transcription – synthesis of DNA on viral RNA template by reverse transcriptase enzyme.

Further division of viruses within the groups is possible by viral type of symmetry, presence or absence of envelope, etc.

It is established to date that above 300 viral species that pertain to more than 30 families can cause human diseases. The current list of viral human pathogens is only expanding all the time.

The viral families of most medical relevance are presented in Table 9.

Table 9
Viral families of major clinical significance

Baltimore group	Type of nucleic acid	Type of symmetry	Envelope	Viral families of medical relevance
I	dsDNA linear	Icosahedral	Non-enveloped	*Adenoviridae*
	dsDNA linear	Icosahedral	Enveloped	*Herpesviridae*
	dsDNA linear	Complex	Enveloped	*Poxviridae*
	dsDNA circular	Icosahedral	Non-enveloped	*Polyomaviridae* *Papillomaviridae*
II	ssDNA linear	Icosahedral	Non-enveloped	*Parvoviridae*
	ssDNA circular	Icosahedral	Non-enveloped	*Anelloviridae*
III	dsRNA segmented	Icosahedral	Non-enveloped	*Picobirnaviridae* *Reoviridae*
IV	ss (+) RNA	Icosahedral	Non-enveloped	*Picornaviridae* *Astroviridae* *Caliciviridae* *Hepeviridae*
	ss (+) RNA	Icosahedral	Enveloped	*Togaviridae* *Flaviviridae*

	ss (+) RNA	Helical	Enveloped	*Coronaviridae*
V	ss (–) RNA	Helical	Enveloped	*Paramyxoviridae* *Filoviridae* *Rhabdoviridae* *Bornaviridae*
	ss (–) RNA segmented	Helical	Enveloped	*Orthomyxoviridae*
	ss (–) RNA segmented	Helical or icosahedral	Enveloped	*Bunyaviridae*
	ss (–) RNA segmented, ambisense	Not determined	Enveloped	*Arenaviridae*
VI	ss (+) RNA 2 copies, reverse transcription	Cone-like	Enveloped	*Retroviridae*
VII	dsDNA circular; reverse transcription	Icosahedral	Enveloped	*Hepadnaviridae*

The note: double-stranded nucleic acid is designated as **ds**, single-stranded – as **ss**.

For individual precise identification of viruses especially isolated from the clinical source further ***infraspecies division*** of viruses is required.

Nevertheless, ICTV states that viral division below the rank of species is not regarded as a part of official taxonomy of viruses.

According to ICTV, the classification and naming of ***serotypes***, ***genotypes***, ***strains***, ***variants*** and ***isolates*** of the same virus species "...is the responsibility of acknowledged international specialist groups".

Particular types and variants of the same virus can be greatly different in their virulence against the human host.

Serotype of virus is determined by reactions with specific antiviral ***antibodies***, e.g. by neutralization tests, ELISA, immunofluorescence assay, complement fixation test, inhibition of hemagglutination, etc.

Genotype of virus (its genetic variant) is identified by ***molecular genetic tests***, e.g. nucleic acid ***hybridization***, ***PCR***, or nucleic acid ***sequencing***.

Viral ***isolate*** is a pure viral culture isolated from the certain patient in the course of infection.

Viral ***strain*** is defined as geneticaly homogenous population of viruses sharing the number of established common characters that distinguish this strain from other viral populations of the same species.

After detailed identification the strain is designated with number, date, point of isolation, etc. and stored in viral collection.

It is generally ascertained that viral members of the same strain share genetic identity at least more than 90%.

The ***variant*** of the same virus usually displays even higher genetic relatedness – about 96-98%.

Nevertheless, in many cases the viruses demonstrate low genetic stability due to the elevated frequency of mutations and recombinations.

During the course of infection the primary virus that initially affected the host undergoes ***rapid individual evolution***. It results from intensive viral replication with multiple mutations as well as from the action of host immune system and/or administered antiviral treatment.

This leads to formation of so-called viral "***quasispecies***". It is not a taxonomic category.

In general, ***quasispecies*** is a total number of closely related genetic variants arisen from intensive mutations of primary virus ("***mutant cloud***") in the course of individual viral infection.

Generation of quasispecies is typical for fast propagating RNA-containing viruses with ***error-prone replication*** with high mutation rate, e.g. hepatitis C virus or HIV.

The emergence of quasispecies is extremely significant in progression of viral infection. The ongoing pressure of host immune system and the influence of antiviral drugs promote the selection of resistant viral mutants. This creates new genetic lines resistant to antiviral therapy and therefore, leads to chronic viral disease.

General Structure of Viruses

Outside the cell the virus stays as inert ***particle*** known as *virion*. It can't propagate in the environment.

Virion *is the extracellular form of virus existence*. Virions are liberated from the cells after virus maturation.

The inner (or core) part of virion harbors viral genome – the molecule of nucleic acid.

Viral nucleic acid is enwrapped with protein ***coat***, termed a ***capsid***.

Capsid is composed of identical protein subunits called ***capsomeres***.

Viral capsid proteins are bound tightly to genomic nucleic acid. This complex is termed ***nucleocapsid***. Great number of viruses contains the capsid as the only external coat. These viruses are determined as the ***naked*** (or ***simplex***) viruses.

More composite viruses carry additional outer lipid membrane or ***envelope***, which is usually obtained from the cytoplasmic membrane of the host cell. These viruses are designated as ***enveloped viruses***.

Lipid coat usually contains the protein termed the ***matrix protein***. Viral glycoprotein receptors form ***spikes*** that jut out of the viral envelope. They are responsible for the viral attachment to the susceptible cells.

Viral structure is organized according to different ***types of symmetry***. Widespread is ***icosahedral*** (or ***cubic***) ***symmetry***, because it is based on the icosahedral pattern – polyhedron with 20 faces. This assemblage provides the most efficient fit of capsomers into the tightly packed coat. Virions of icosahedral symmetry are usually ***spherical*** in shape.

Another type is ***helical symmetry***, where protein subunits with core nucleic acid are wound into the common helix with periodic coils. This complex is packed within the external lipid envelope.

And some viruses with composite architectonics possess ***complex structure*** (or ***mixed symmetry***). For instance, bacteriophages resemble spermatozoids in their shapes, where phage head is of icosahedral symmetry and the tail is of helical symmetry. Poxvirus resembles brick in shape with lateral bodies within the envelope.

Naked viruses are usually resistant to the ether or other organic solvents, whereas enveloped lipid-containing viruses are sensitive. Impairment of external lipid shell leads to the loss of viral infectivity.

There is a very special group of viruses, composed solely of nucleic acid, termed ***viroids***.

Viroids are the smallest viral agents infecting plants. Known viroids contain only the small single-stranded circular RNA. They are devoid of capsid and viroid RNA doesn’t encode protein molecules.

Chemical Structure of Viruses

Virus particles are composed of different kinds of polymeric molecules.

Viral proteins are divided into ***structural*** and ***non-structural***.

Supercapsid and ***capsid*** proteins are referred to ***structural proteins***.

Supercapsid proteins are subdivided into ***attachment*** and ***fusion*** proteins.

Attachment proteins initiate the recognition of susceptible cell by virus and provide specific interaction of virion with cell surface receptors.

Attachment glycoproteins are presented in viral spikes that bind to the target cells (e.g. hemagglutinin of influenza virus).

Fusion proteins accelerate viral fusion with the cell membrane.

Capsid proteins form nucleocapsid protein units (capsomers).

Non-structural proteins comprise the ***early precursors of viral proteins***; ***viral enzymes*** (DNA- and RNA-polymerases, proteases, neuraminidase, etc.); ***genomic*** and ***regulatory proteins***, which control transcription and translation.

Most of non-structural viral proteins are expressed inside the cells in the course of viral infection.

Viral proteins possess antigenic properties, reacting with specific antibodies and immune cell receptors.

Besides its own proteins, during maturation the virus may capture the proteins of the host cells. For instance, HIV harbors cellular protein cyclophilin up to 30% of its weight; without this protein HIV particles are non-infectious.

Viral ***lipids*** are obtained from the infected cells, coming into the structure of viral envelope. They provide viral infectivity and stimulate hydrophobic interaction of virus particles with cell membranes facilitating viral entry. Overall, lipid structures comprise up to 30% of enveloped virion mass.

Carbohydrate residues are usually bound to proteins in glycoprotein complexes of spikes and other viral receptors. They cover about 10-15% of virion contents.

Viral Genomic Organization

As was previously mentioned, viral genome includes a single type of nucleic acid. Viral DNA or RNA may be single-stranded (e.g. ortho- and paramyxoviruses) or double-stranded (reoviruses), segmented (orthomyxoviruses, etc.) or non-segmented (togaviruses, picornaviruses and many others), positive or negative, circular or linear.

Protein-encoding strand of nucleic acid is called ***positive***, or ***plus-strand***. This strand serves as a direct template for subsequent transcription and translation.

RNA(+)-containing viruses (picornaviruses, togaviruses) use positive-sense nucleic acid as a messenger RNA for protein synthesis. This RNA is considered to possess the ***infectivity***, being able to induce the infectious process directly after virus penetration.

Negative or ***minus strand*** means the nucleic acid chain complementary the positive one. In that case viral protein formation is impossible without preliminary synthesis of positive chain on the negative strand template. For this purpose (–) RNA viruses contain RNA polymerase. It catalyzes the complementary RNA(+) synthesis within infected cells. The latter serves as mRNA.

The smallest DNA-containing organisms are hepadnaviruses with genome size of 3.2 kbp, the largest poxviruses have the genome of 375 kbp.

Retroviruses carry ***reverse transcriptase***, an enzyme that performs single strand DNA copy of viral RNA template. Then a second complementary strand of DNA is polymerized. This DNA molecule is capable of further integration with DNA of the infected animal or human cell, and retrovirus comes into ***provirus*** state. Integrated DNA serves as a template for transcription and translation of retroviral proteins.

Poxviruses are the most intricately constructed viruses. Virions carry different enzymes in their own transcriptional system for nucleic acid and protein synthesis.

Nucleic acid structure of human pathogenic viruses of the main importance is presented in the Table 9.

Virus replication cycle

Viral ***life cycle*** passes through definite consecutive stages:
– viral attachment;
– penetration (virus entry);
– uncoating;
– biosynthesis of viral components;
– morphogenesis with assembly of viral particles;
– virion release (or egress).

The first step of infection is the ***attachment stage*** with ***adsorption*** of virions to the host cells. Viral ***spikes***, containing ***attachment proteins***, project out of the surface of the viral shell. Viral receptor molecules as well as the opposite cell receptors are usually glycoproteins. In some cases virus interacts with cell protein sequences (picornaviruses) and with oligosaccharides (orthomyxoviruses or paramyxoviruses).

Cell receptor density for particular virus is in the range 10,000-100,000 per infected cell.

Viral penetration or ***virus entry*** involves different mechanisms, depending on virus nature. Naked viruses are captured into the host cell by ***endocytosis*** (or *viropexis*) after virus adsorption. Adsorption usually occurs in membrane sites enriched with cellular receptor proteins *clathrin* or *caveolin*.

Virion-containing vesicle (***endosome*** or vacuole) is opened in cytoplasm, disseminating viral particles.

Enveloped viruses can use the endocytosis mechanism also. The fragment of the cell plasma membrane enwraps the attached virion with the vesicle formation. Further the virus lipid envelope fuses with the cell membrane due to hydrophobic forces with subsequent release of free nucleocapsids into the cytoplasm.

In another case the direct fusion of viral envelope with the plasma membrane is performed following the strong specific adsorption of viruses to the host cell receptors. Specific ***fusion proteins*** stimulate membrane fusion (e.g. F-protein of paramyxoviruses, the similar action is controlled by influenza virus hemagglutinin). Fusion proteins promote specific type of viral ***cytopathic activity*** (see below), causing host cell integration with symplasts and syncytium appearance. The envelope-devoid nucleocapsids are then liberated into the cytoplasm under the inner side of the cell membrane.

Uncoating of virus results in nucleic acid release from the surrounding proteins before the genome replication and early protein synthesis. Uncoating follows viral entry and continues after penetration. Dissolution of virus is facilitated by acidic pH in the endosome. Viral genome can be liberated as a naked nucleic acid (property of picornaviruses) or as a nucleocapsid (essential for reoviruses). In latter case it carries polymerases, necessary for further viral replication.

Biosynthesis of viral components varies strongly in different viruses. It depends on viral nucleic acid structure and polarity.

DNA viruses are reproduced usually in the nucleus of the infected cells. They use cell DNA and RNA polymerases for nucleic acid replication.

The majority of DNA viruses contains double-stranded DNA, which is transcribed into sense mRNA (e.g herpesviruses). The latter is used as a pattern for protein synthesis.

The final transcript of mRNA can be achieved in several ways including reading frame shift or change of transcription starting point in the same reading frame (***overlapping genes***).

Splicing of primary transcript, where the elimination of inserted non-coding RNA fragments occurs resulting in formation of mature mRNA, is essential for adenoviruses.

Newly formed viral mRNAs are translated on cellular ribosomes yielding viral proteins.

Virus-induced ***early viral proteins*** are synthesized before the replication of viral genome. They are produced in host cell ribosomes usung viral mRNA template. Most of them are viral enzymes and regulatory protens serving for the next steps of viral reproduction.

Late proteins are mostly the structural units of viral capsid; they are formed after the replication of viral genomic nucleic acid.

Replication patterns of ***RNA viruses*** are even more entangled.

For instance, reoviruses use initial double stranded segmented RNA for mRNA synthesis by the own viral RNA polymerase.

Positive single-stranded RNA of many viruses (e.g. picornaviruses or flaviviruses) is infectious, and it is used as a template for direct protein synthesis. Viral RNA of these viruses is multiplied through double-stranded plus-minus RNA intermediate.

Viruses, containing ***negative genomic RNA*** (e.g. rhabdoviruses, paramyxoviruses, orthomyxoviruses), synthesize positive RNA strand by viral RNA polymerase. In case of ***segmented viral genomes*** (orthomyxoviruses) mRNA is transcribed sequentially from different segments.

Long replication cycle of ***retroviruses*** is maintained by viral ***reverse transcriptase***. It catalyzes DNA copy formation on the viral RNA template. After DNA integration into the host cell genome it is used for transcription of mRNA, coding for the viral proteins.

Viral genomic RNA is usually multiplied in the cytoplasm of infected cells with some exceptions (e.g. retroviruses).

RNA-containing viruses are characterized by the almost simultaneous expression of all viral proteins. Some viruses (picornaviruses, retroviruses) translate mRNA into common precursor polyprotein, which is cleaved by proteases with final formation of protein sequence.

Overall, viruses demonstrate the ***disjunctive type of reproduction***, where viral components (DNA and proteins of the coat) are synthesized in separate bacterial cell compartments.

Viral morphogenesis (or *maturation* stage) includes the ***self-assembly*** of virions within the cells. It is the multistep process of viral capsid formation and nucleic acid packing.

The time interval between the virus penetration and the end of viral assembly is known as the ***eclipse period***, where the virus is deprived of infectivity, being incapable of causing infectious process. The infectivity is restored only after full-value maturation of virus progeny.

Virion release (or viral ***egress***) is performed in several ways. In case of the cell death due to viral infection the virus is liberated by the ***lysis*** of the host cell. Another mechanism is ***budding*** through the cellular membrane, which retains the viability of the infected cells.

For enveloped viruses the maturation step is accomplished during budding, where the fragments of plasma membrane cover the nucleocapsid making viral ***envelope*** with parallel embedding of matrix proteins and spikes.

The whole length of the virus replication cycle varies from 6-8 hours for picornaviruses to several days for adenoviruses or measles viruses.

Sometimes after reproduction cycle the ***defective viruses*** are formed, which are usually non-infectious. Such virus particles lack some important genes due to incorrect nucleic acid excision or impaired viral protein translation and assemblage.

Outcomes of viral infection result in the ***productive***, ***persistent***, ***transforming*** and ***latent*** infection.

Productive infection leads to active accumulation of viral particles with destruction of infected cells e.g., by lysis. Newly synthesized virions are able to spurt the infection, penetrating into neighboring susceptible cells. In most of cases it is characteristic for ***acute*** viral infections.

Persistent infection progresses much more slowly. It might be followed by low viral replication with slow budding from the infected cells. Thus, the host cells survive and can propagate. It is related with ***chronic*** viral infection

Latent virus infection evolves, when the virus is continuously present inside the infected cells, but its reproduction is very slow or even ceased.

Likewise, latent infection is established after the integration of the viral genome into the cell DNA with formation of provirus (***integrative infection***). In that case mature virus particles are not produced for a long time.

Latent infection also corresponds to chronic viral disease.

Transforming infection is promoted by particular type of viruses (e.g. *Oncovirinae* subfamily representatives, papillomaviruses, etc.) Transformation is mediated by complex chain of genetic events, caused by virus, which affect the genome of susceptible cells. The virus can initiate tumor growth in the infected organism.

Laboratory Diagnosis of Viral Infections. Methods for Virus Cultivation

For ***laboratory diagnosis*** of viral diseases the ***indication*** and ***identification*** of certain virus present in clinical specimen is elaborated.

Indication of viruses means the discovery of virus presence in the clinical material, whereas ***virus identification*** assumes the exact determination of virus species or ***type*** (primarily, viral ***serotype*** and viral ***genetic variant*** or ***genotype***).

Three basic groups of laboratory tests are used in clinical practice for indication and identification of viruses:

– ***rapid*** (or express) tests allowing ***direct detection*** of viruses in clinical specimens;

– ***isolation of viral culture***;

– ***serological*** tests that confirm the diagnosis of viral infection by detection of ***specific antibodies*** against viral antigens in patient's serum.

As current viral taxonomy is primarily based on genetic ground, the precise determination of viral genetic variant (genotype) is pivotal for correct identification of virus. For this purpose versatile ***molecular genetic tests*** like methods of nucleic acid ***sequencing***, ***PCR*** and nucleic acid ***hybridization*** are commonly used.

PCR is the most convenient and reliable as well as sensitive and specific genetic test with possibility of full automation that is available now for clinical practice.

On the other hand, ***DNA microarray*** technologies based on parallel hybridization of multitude of nucleic acid probes placed in ***DNA biochip*** allow simultaneous testing of hundreds of clinical specimens for specific viral DNA or RNA. This creates excellent opportunities for mass screening of population for viral infections.

Finally, highly sophisticated methods of nucleic acid ***sequencing*** play the role of the reference tests for precise identification of viruses.

Serotype of virus is determined by reactions with specific antiviral ***antibodies***, e.g. by neutralization tests, ELISA, immunofluorescence assay, complement fixation test, inhibition of hemagglutination, etc.

PCR and molecular ***hybridization of nucleic acids*** as well as ***ELISA*** and ***immunofluorescence assay*** are the most commonly used methods for ***rapid*** detection of virus ***directly in clinical specimen***.

Genetic tests made possible the identification of fastidious viruses that can't be cultured in laboratory cell lines – PCR or nucleic acid probing reveals nucleic acids of these viruses directly in the host tissues. For instance, noncultivatable sarcoma Kaposi virus was detected in tissues of AIDS patients and was proven to be the herpes family representative (herpesvirus type 8) by methods of molecular genetic analyses.

For ***isolation of virus cultures*** (as the viruses can propagate only inside the living cells) three main models are commonly used: laboratory ***cell cultures***, ***chicken embryos*** and ***susceptible laboratory animals***.

The main ***aims*** of virus cultivation presume the efficient laboratory diagnosis of viral infections; investigation of pathogenesis of viral diseases; and laboratory design and trials of antiviral drugs and vaccines.

The most simple and cheap model of virus propagation is their cultivation in ***embryonated chicken eggs*** under strictly controlled conditions. Virus-containing material can be inoculated in any compartment of the embryonated egg.

Indication of viral growth in the fertile chick eggs is performed by estimation of embryo death, vessels impairment, production of pocks or plaques on the chorioallantoic membrane (e.g., it is characteristic for herpes, smallpox, or vaccinia viruses).

Viruses, expressing hemagglutinins in their external coat, are shown to induce ***hemagglutination*** and ***hemadsorption*** phenomenons, clumping erythrocytes of different animal species. This is essential for ortho- and paramyxoviruses, certain types picornaviruses, and others.

As the result, after cultivation in chicken embryo the ***indication*** of hemagglutinating virus is performed by ***hemagglutination test***. In this case the twofold dilutions of the allantoic fluid are mixed with sensitive red blood cell suspension. During the incubation the virions interact with erythrocytes, mediating their aggregation. The surface viral hemagglutinins of virion can attach simultaneously to two and more separate red blood cells that promote erythrocyte clumping.

The ***endpoint of hemagglutination test*** (or ***virus titer***) is determined as *the greatest dilution of the virus-containing material, which causes a clearly marked hemagglutination*.

Similarly virus indication by hemadsorption is performed using the microscopy of the mixture of virus-infected embryo cells with erythocytes. In latter case the eukaryotic infected cells, surrounded by adsorbed erythrocytes, are readily discernible in the smear.

Identification of hemagglutinating virus in the chicken embryo is carried out by *virus* ***hemagglutination inhibition test***. After primary

incubation of the virus-containing allantoic fluid with the dilutions of antiviral type-specific antibodies, the erythrocyte suspension is added. As antibodies block the viral hemagglutinins, the hemagglutination is inhibited, and the virus serotype is determined.

Nevertheless, the most suitable and widespread model of virus investigation is the viral ***cultivation*** in ***cell*** (or ***tissue***) ***cultures***.

All cell cultures are prepared from animal or human source. The tissue is minced into small pieces and homogenized. Then it is treated by proteolytic enzyme (mainly, by trypsin or collagenase) to disintegrate intercellular matrix. The cell suspension is placed into culture multiwell plate or flask with sterile nutrient complex medium, containing all necessary growth factors, such as amino acids, carbohydrates, vitamins, salts and sometimes fetal serum components and antibiotics to prevent bacterial growth. The cells propagate in the medium and finally form a single layer of the cells (***cell monolayer***), attached to the plastic surface.

There are three main types of cell cultures: ***primary***, ***secondary*** or ***diploid***, and ***continuous***.

Primary cell cultures are obtained from different human and animal tissues (human skin fibroblasts, monkey kidney cells, etc.) They grow for 1-3 weeks with final autolysis and culture death. In most of cases primary cultures undergo only 1-2 passages, thus they are not suitable for long-term cultivation.

Cell *passage* (or *subculture*) means reinoculation of a small portion of basic cell culture grown in the medium into another well or flask with fresh nutrient medium for further propagation. Cell passaging substantially expands the survival time of laboratory cell lines.

Diploid (or *semi-continuous*) ***cell cultures*** are designed for longer cultivation. The sources for diploid cell lines are fetal tissues (e.g., human diploid lung fibroblasts, human breast epithelial cells, etc.) They are also of limited survival, but can propagate for 40-50 or more passages. The nuclei of diploid cells retain their normal chromosome pattern. They can be used for cultivation of most viruses.

And ***continuous cell cultures*** are originated from the almost immortal tumor cell lines (***HeLa*** cells from human cervical cancer, HEp-2 from larynx carcinoma, McCoy cells, and many others.) Tumor cells can divide indefinitely long *in vitro*. These lines are appropriate for many viruses, but their genome might be not completely stable, rendering rare but cumulating chromosome aberrations. Also they can be occasionally contaminated by bacterial intracellular parasites (e.g., mycoplasmas) in course of long-time cultivation.

Viral ***indication*** and ***identification in the cell cultures*** is carried out by several methods.

Viruses are ***indicated*** by their ***cytopathic effect***. During cultivation the viruses impact on the cell life cycle resulting in changes of cell morphology and viability. This viral action is known as ***cytopathic effect***.

Different types of cytopathic effects exist – degeneration and destruction of monolayer, cell lysis or necrosis, ***plaque*** appearance, ***inclusion*** formation, cytoplasmic vacuolization, ***symplast***, ***syncytium*** and giant cell formation. Many viruses develop a special cytopathic effect, which is used for viral indication.

Inclusion formation is the characteristics of many viruses. RNA viruses (e.g. rhabdoviruses) usually form inclusions in the cell cytoplasm, while DNA-containing agents (e.g. herpesviruses) – in the nuclei of infected cells. However, the cells, infected with DNA-containing poxviruses, produce the specific inclusions (***Guarniery bodies***) in cytoplasm. Some viruses are shown to form inclusions both in the cell nucleus and cytoplasm (measles virus). Inclusions are the sites of virus intracellular replication.

Different viruses can induce ***symplast*** and ***syncytium*** formation in the cell cultures because of virus-induced cell fusion activity. Giant polynuclear epithelial cells (***symplasts***) are formed after measles virus action. Respiratory syncytial virus and HIV exert the damage of infected cells by ***syncytium*** formation.

Plaques appear after cell monolayer destruction and lysis, caused by virus. Plaques look like clear zones within the cell culture. This method is used for the determination of virus quantity, because each plaque is the result of single virus particle propagation within susceptible cells. Total virus count is calculated by multiplication of plaque quantity by the dilution of the sample.

Indirect evaluation of viral cytopathic activity is performed by ***color reactions*** in the cell cultures. Test tubes, containing cell cultures, virus sample, and nutrient medium with indicator dye, are incubated for several days. During incubation the virus replicates within susceptible cells and destruct them. Control test tubes are free of virus, and the abundant growth of the cell culture is observed. In the control tubes the color of the indicator medium is changed due to the accumulation of products of cellular metabolism, resulting in the medium acidification and pH lowering. And vice versa, viral cytopathic activity blocks cell metabolism retaining the initial color of the medium.

Hemagglutination and ***hemadsorption*** are also used for hemagglutinin-containing virus indication in the infected cell cultures. Influenza and parainfluenza viruses are determined by these reactions.

Viral identification in the cell and tissue cultures is based on ***virus neutralization reactions***, promoted by specific antiviral antibodies, which block viral activity. The inhibition of virus hemagglutination and hemadsorbtion, neutralization of plaque and inclusion formation, neutralization of color reactions can be applied.

And obviously, for precise indication and identification of viral isolates grown in cell culture lines, all ***genetic*** methods (like ***PCR*** or nucleic acid ***hybridization***) or ***serological*** tests (immunofluorescence or ELISA test) are commonly used in clinical virology laboratories.

Tissue and cell cultivation makes possible the rapid detection and diagnosis of viral infections. Also it is the suitable technology necessary to obtain the pure viral culture from cell lysate. It can be used for virus diagnosticum production or vaccine design.

Sometimes the viruses are inoculated to propagate in the ***susceptible laboratory animals***. For instance, Coxsackie viruses infect newborn "suckling" mice with characteristic disease development. Indication and identification of viruses in this model is similar with the above described.

Serological testing allows to confirm the diagnosis of viral infections by detection of ***specific viral antibodies*** arisen in patient's serum. A broad group of serological methods are currently used in laboratory practice – ***ELISA*** (the most common test), hemagglutination inhibition assay, radioimmunoassay, complement fixation test, neutralization test and some others.

Identification of specific antibodies of IgM class indicates acute primary viral infection

In many clinical situations serological ***paired sera test*** is elaborated, where patient's antiviral antibodies are tested at least twice – early after the disease onset and next closer to patient's recovery. The ***fourfold increase*** of the titers of specific antibodies gives the confirmation of current viral infection.

Chapter 14
BACTERIOPHAGES

Bacteriophages, the History of Discovery and Initial Characteristics

Bacteriophages *are viruses, which are specific parasites of bacteria*.

It is generally acknowledged that bacteriophage activity was described first by F. d'Herelle in 1917. He discovered the specific lysis of bacterial culture affected by non-cellular filtrate of dysentheria patients feces. However, the similar phenomenon was observed previously by N. Gamaleya in 1892 and F. Twort in 1915.

Bacteriophages are ubiquitous in nature. They accompany bacteria in any place of their habitation: animal or human body, plants, water, soil, drainage waters, etc. For instance, phages are essential component of ***bacterioplankton,*** the bacteria found in the ocean. In this environment up to 2.5 x 10^8 phages/ml have been found.

Similar way bacteriophages are commonly isolated from different bacteria-containing foodstuffs (dairy products, fruits, etc.)

In patients, suffering from various bacterial diseases, the phages are excreted by feces, urine, sputum, and saliva in the parallel with bacterial discharge. They are important ***epidemiological markers*** of the bacterial infection.

Morphology of Phages

Bacteriophages are typical viruses of ***complex structure***.

The majority of phages resembles spermatozoids in their shapes but filamentous and some other forms occur. Head of phage contains viral genome – DNA or RNA.

The phage size usually varies from 20 to 200 nm.

Main structural components of phages are nucleic acid (DNA or RNA) and proteins. Bacteriophage RNA is single-stranded. DNA may be single or double-stranded. Nucleic acids of phage are tightly packed into the phage's head.

Bacteriophages are composed of ***head***, protein ***tail*** with hollow core and ***fibers***, attached to tail ***basal plate***.

Phage head is of protein nature with ***icosahedral symmetry***. It can be naked or covered with ***external envelope*** (***supercapsid***). Phage head can

carry ***spikes*** with receptor activity. Inner head proteins support DNA supercoiling. Phage's tail of ***helical symmetry*** is covered with ***sheath***, composed of contractile proteins, which provide tail contraction. They can be connected with calcium ions, and different enzymes (e.g., endolysins, ATPase or some others).

Small amount of lipids are also present in phages envelope.

Total protein content is about 50% of phage weight, nucleic acid content – 40-50% and lipids are about 1.5-3%.

Well-studied is a group of ***T-*** (or ***type***) phages of *E. coli*, as well as temperate *E. coli* lambda-phage, some filamentous phages (for instance, M13 phage) and many others.

The differentiation of phages is based upon type of nucleic acid, phage's morphology, chemical structure, and their interaction with the bacterial cell.

As the result, all phages are divided into ***DNA-*** and ***RNA-containing***.

According to their ***morphology*** they are separated into several types:

– DNA filamentous phages;
– RNA-containing phages with rudimentary tails;
– DNA phages with short tails;
– DNA phages with long non-contractile tails;
– DNA phages with long contractile tails.

Phages are enough resistant to different physical and chemical factors (radiation, drying, pH fluctuations, or temperature). Nevertheless, they are rapidly inactivated by boiling, chemical disinfectants, UV-light.

Interaction of Phages with Bacterial Cells

There are four basic stages in phage-bacterial interaction that generally reflect the common steps of any viral replication cycle: ***adsorption***, cell ***penetration***, phage ***reproduction***, and ***release*** of newly formed phage particles.

Adsorption is provided by the attachment of receptor fiber proteins of the phage tail to the specific receptors on the bacterial cell wall.

Reversible and irreversible phases of adsorption are indicated. The adsorption is accelerated by divalent cations (Ca^{2+} and Mg^{2+}).

Penetration is simulated by the action of phage enzymes. For instance, phages T4 of *E. coli* possess lysozyme-like enzyme ***endolysin***, which degrades the minimal site of the bacterial cell wall. The tip of the

tail opens allowing viral DNA to move through the channel of the phage tail. Phage ATPase generates energy for tail contraction, and genomic nucleic acid is injected by into bacterial cell, passing through the cell wall and cytoplasmic membrane. The phage capsid remains outside the cell.

Reproduction stage covers the period between the phage penetration and release of newly created phage particles. At the beginning of reproduction there is short-term *eclipse phase*, when phage biopolymers (genomic DNA and proteins of the coat) are synthesized.

Phage DNA serves both as the template for replication of new phage DNA molecules and for transcription of matrix RNA, which encodes phage proteins.

After the DNA penetration a number of ***"early" enzymes*** and other proteins are formed within the cell. They are generally termed as ***phage-induced proteins***. Their synthesis occurs due to the partial transcription of phage DNA by cellular polymerases. Some of these proteins (for instance, ***phage-induced nuclease***) block bacterial cell DNA replication, and the phage switches bacterial intracellular machinery to its own purposes.

As the result, the enzymes of bacterial cell actively supply the process of phage reproduction with energy and monomers for protein and nucleic acid synthesis. Bacterial ribosomal apparatus produces phage proteins.

Then the ***assembly*** of structural components of phage (its head, tail and tail spikes, fibers) is activated.

Late-induced enzymes provide the assembly of phage particles and phage release out of bacterial cell.

Phage assembly is a complex process. After phage head formation DNA is packed into it, then the tail is attached with subsequent addition of tail spikes.

Overall, for above mentioned T4-phage eclipse phase is finished within about 30 minutes; and new viable phage particles can be detected inside the bacterial cell.

Phage release is initiated by late-induced enzymes with cell wall hydrolyzing activity (***endolysins*** or murein hydrolases). They break down bacterial peptidoglycan layer with subsequent phage dissemination.

Finally, hundreds of newly formed phage particles are liberated, and these virions are capable of infecting the neighbouring bacterial cells.

Outcomes of Phage Infections

The infectious process arisen in bacterial cells by specific bacteriophages can be resolved in various ways.

Virulent phage infection leads to the burst maturation of new phages with further lysis of the bacterial cell. In this case the cycle of phage propagation lasts for 30-90 minutes. It is the variant of ***productive viral infection*** that is followed by host cell disruption with massive virion egress.

Lysogenic infection or ***lysogeny*** is caused by ***temperate phages***. These phages can integrate its DNA into the nucleoid of bacterial cell. Phage DNA then replicates as an integral part of the bacterial chromosome.

Nucleoid-integrated bacteriophage is known as a ***prophage***, while the bacterial cell infected with prophage is called a ***lysogenic cell,*** or ***lysogen.***

The well-known example of temperate bacteriophage is the ***phage lambda***, which interacts with *E. coli.*

Phage DNA integration into the bacterial chromosome is possible due to ***site-specific recombination*** between bacterial and phage genomes. Short nucleotide sequences in the DNA of phage and host nucleoid are ***homologous***, allowing closest phage and bacterial DNA interaction (***synapse***) with subsequent genomes consolidation (for details see the Chapter of bacterial genetics).

Being integrated, the phage DNA can remain in the prophage state for a long period of time. Its expression can enrich the host cell with some new properties. This bacterial cell state is known as ***lysogenic (or phage) conversion***. Very often conversed bacterial cells become virulent after phage acquisition. For instance, *Corynebacterium diphtheriae*, *Clostridium botulinum* and *Vibrio cholerae* start to express extremely poisonous exotoxins. That is true also for beta-hemolytic streptococci, which acquire the ability to produce erythrogenic toxin that actively participate in scarlet fever, and for salmonellae that change LPS structure of cell wall due to phage infection.

From the other hand, if prophage disappears from bacterial cell, the cell becomes avirulent.

In the period of lysogeny the infected cell acquires the immunity to a certain type of phage. ***Repressor protein***, encoded by one of integrated viral genes, blocks possible transformation of prophage into active virulent form.

Bacteriophage activation with excision of viral genome (***phage induction***) is usually an accidental low-incidence process. It is occurred

after lysogenic bacterium exposure to ultraviolet light, irradiation, chemicals action, etc. In that case the prophage transforms into a mature vegetative phage.

Sometimes after non-proper excision temperate bacteriophages can capture the bacterial genes surrounding phage nucleic sequence. In that case the phage becomes ***defective*** but able to transfer different host bacterial genes to other susceptible bacteria.

This phenomenon is known as ***transduction***, and it makes available the exchange of genetic material between bacterial cells. This results in great raise of bacterial population diversity, which is valuable for microbial evolution. Molecular transduction is actively used in genetic engineering.

Filamentous phages can leave the infected cells without their destruction. All of them are of single-stranded DNA genome. Filamentous phages release is known as ***extrusion*** from the bacterial cell. In that case infected bacteria save their ability to cell division. Infected bacteria are called as ***carrier cells***.

Finally, about 50% of bacterial species are proven to carry special genetic region responsible for so-called bacterial "***adaptive immune system***". This system maintains the specific protection of microbial cells against bacteriophage infection. For instance, it is essential for many aggressive human pathogens like *M. tuberculosis* or *Y. pestis*.

Genome of these bacteria contains the number of genetic elements known as ***CRISPR cassettes*** (abbreviation "CRISPR" means "*clustered regularly interspaced short palindromic repeats*").

CRISPR cassette includes many short genetic ***spacers*** of similar length but ***of different DNA sequence*** interspersed between almost identical direct repeats of DNA. A single CRISPR cassette may contain more than 100 spacers. Every spacer harbors a short sequence of foreign DNA captured by bacterial cell during the previous infection with bacteriophage or plasmid.

The genetic elements of this system control the acquired defensive reactions of bacterial cell against the invaded foreign nucleic acid of bacteriophage. Furthermore, the genetic information of invaded DNA is memorized in bacterial genome and becomes heritable. As the result, next entry of the same nucleic acid of bacteriophage leads to the activation of specific RNA-mediated enzymatic reactions that actively eliminate the extraneous nucleic acid of phage.

The family of bacterial enzymes with ***integrase*** and ***nuclease*** activity generally termed as ***Cas proteins*** are responsible for uptake and degradation of phage nucleic acids. They are encoded by special bacterial

genetic locus ***cas*** (*cas* means *CRISPR-associated*) that borders with CRISPR genetic region.

The details of functioning of bacterial CRISPR/Cas systems were presented earlier in the section of microbial genetics (Part 1 of the textbook).

Production of Phage Culture

To obtain virulent bacteriophages, the susceptible bacterial cells are infected by minimal amount of phage particles. During incubation at 37^0C for 18-24 h phage population propagates causing bacterial cell lysis. The remaining microbial fragments are removed from phage culture by centrifugation or filtration. The filtrate is tested for purity, sterility, and biological activity.

Laboratory Determination of Bacteriophage Activity

Bacteriophage presence in the investigated sample can be revealed by drop of the filtrated material on Petri dish with appropriate medium, pour plated with the susceptible bacterial culture. After incubation for 24-48 h at 37^0C the infection of bacteria by phages results in a ***plaque*** formation, a clear area in the bacterial lawn. The plaque is formed due to phage lytic activity.

Quantitative phage determination is possible by ***titration methods***.

Two main variations of these methods are based on phage ***titration*** in ***liquid*** or ***solid nutrient medium***.

In first case tenfold dilutions of the phage-containing material are prepared and then inoculated into liquid nutrient medium. The susceptible microbial culture is added to the broth. After incubation for 24-48 h at 37^0C the growth inhibition is evaluated. The ***phage titer*** is established as the last dilution of the phage culture, which is able to cause complete inhibition of visible microbial growth.

Similar ***agar titration method*** is performed as follows: mixture of susceptible bacterial culture with tenfold phage dilutions is poured on different Petri dishes with solid nutrient medium. After incubation for 24-48 h at 37^oC the growth inhibition with ***plaque*** formation is estimated. The last phage dilution resulting in isolated plaque formation is considered to be the ***endpoint*** (***titer***) of the reaction. Total phage quantity is calculated by multiplication of last plaque count by the dilution of the sample.

Practical Applications of Phages in Biology and Medicine

Practical applicability of bacteriophages in various fields of biology and medicine is based on their capacity to specific interaction with susceptible bacterial strains.

First of all, bacteriophages are long time used for ***laboratory diagnosis*** of a large number of infectious diseases.

Species-specific phages interact with bacteria of certain bacterial species, whereas ***type***-specific ***phages*** can affect only particular variant of bacteria within the same species. This process is known as ***phagotyping***. The specific ***phagotype*** of bacterial strain is proven to be a powerful ***epidemiological marker*** of the infection. Isolation of bacteria of the same phagotype from various human or animal hosts allows to trace the course of infection.

Likewise, phagotyping is used for precise ***identification of bacterial isolates*** of a great number of microbial species (salmonella, shigellae, staphylococci, causative agents of plague, cholera, etc.)

Also bacteriophages are being actively implemented now into medical ***treatment*** and ***prophylaxis*** of bacterial infections. This is primarily related with exuberant spread of multidrug resistant bacteria causing severe hospital-acquired infections (e.g., methicillin resistant *Staphylococcus aureus* strains, isolates of *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, and others).

Finally, phages vectors are widely used in ***genetic engineering*** for design of recombinant vaccines, hormones, cytokines and many other valuable biological products.

Modern advanced vectors include complex genetic systems that contain DNA of various origins; for example, combined phage and plasmid DNA sequences.

Chapter 15

CAUSATIVE AGENTS OF VIRAL RESPIRATORY INFECTIONS

ORTHOMYXOVIRUSES

The History of Discovery of Influenzaviruses

Influenza is known from the Hippocrates era. Further flu-like epidemics were described in Middle Ages. Well-documented are influenza epidemics of 1890 and of 1900. Finally, the global influenza pandemic of 1918-1919 (Spanish flu) caused the death of more than 20 million people.

Viral etiology of influenza was established by W. Smith, C. Andrews, and R. Laidlaw, who isolated influenza type A virus in 1933.

T. Francis and T. Magill isolated influenza type B virus in 1940, and type C influenza virus was discovered by R. Taylor in 1947. Previously circulated pandemic strains were ascertained later as influenza viruses by serological investigations; this conclusion was confirmed nowadays by genetic tests (PCR and others).

Viruses of influenza type A pose the most serious threat for public health. They regularly cause seasonal influenza epidemics repeated in every 2-3 years, but also they can cause severe global ***pandemics*** of the disease. Influenza type B virus usually accounts for seasonal influenza outbreaks at time intervals of 3-6 years, and influenza C virus promotes only mild, sporadic forms of diseases. These differences rely upon the virus type A ability to undergo gene reassortment with subsequent change of viral antigenic structure. Thus, in several decades after the previous pandemic, a new subtype of influenza A arises in human circulation, resulting in new pandemic (e.g. H1N1 in 1918; H2N2 in 1957; H3N2 – Hong Kong flu – in 1968). H1N1 strain returned in 1977 (Russian flu).

It was proven that all of pandemic strains are the reassortants between avian and human influenza viruses, except Hong Kong flu virus.

Last global influenza pandemic later called as “swine flu” was declared by WHO in 2009. It originated from a newly emerged pandemic strain of H1N1 virus designated A/2009/H1N1 (or *pdm09* strain). This virus appeared to be quadruple reassortant of primary human, avian, and pig influenza viruses.

In 1997 the initial case of devastating human infection caused by avian influenza A virus (***H5N1***, ***avian flu***) has been registered in Hong Kong. Since that time the multiple cases of H5N1 avian flu were observed in Southeast Asia with common fatality rate of more than 50%.

Finally, the first human cases of avian flu engendered by novel highly pathogenic avian virus H7N9 were indicated in China in 2013. The lethality of epidemic outbreaks of this disease exceeded 25%.

Classification of Influenza Viruses

The family *Orthomyxoviridae* currently comprises 6 viral genera.

The causative agents of influenza are placed into 3 separate genera *Influenzavirus A, B* and *C,* respectively, each harboring the single viral species of the same names (influenza A, B, or C viruses).

Different viral types A, B, C are distinguished by their serological properties.

A, B, and ***C type division*** of influenza viruses is based on antigenic variations of nucleocapsid ***NP*** viral structural proteins. They are distinct in all three types. Virus ***subtyping*** is performed according to antigenic differences of the surface glycoproteins ***hemagglutinin*** (***H***) and ***neuraminidase*** (***N***). Currently 18 subtypes of H (H1-H18) and 11 of N (N1-N11) are described. They are combined in many variations. Influenza virus strains with five different HA (H1-H3, H5, and H7) and three N (Nl, N2, and N9) were isolated from humans.

The strains of influenza viruses are marked by virus type, host, geographic origin, number and year of the strain isolation and by its H and N numbers, e.g., A/Hong Kong/03/68(H3N2).

Structure of Viruses

Influenza virus of A and B types contains ***single-stranded negative RNA***, which is segmented into 8 fragments (RNA of type C viruses contains 7 fragments without neuraminidase gene). It is lipid ***enveloped*** virus of ***spherical*** shape.

The diameter of viral particles varies in the range of 80-120 nm. Viral nucleocapsid is of ***helical*** symmetry.

Influenza virus A carries at least 15 distinct proteins.

Type-specific ***nucleoprotein*** (***NP***) is tightly bound to viral RNA. Also the virion contains its own ***RNA polymerase***.

Viral ***matrix M1*** protein scaffolds the outer coat of influenza virus; ***M2*** structural proteins make numerous ionic channels in viral coat facilitating disassembly of virion.

The viral particle is enwrapped by lipid envelope of host cell origin with external spikes. Different spikes contain two major glycoproteins – ***hemagglutinin*** (***H***) and ***neuraminidase*** (***N***).

Viral ***H*** is a major viral receptor that binds to sialic acid residues of receptors of upper respiratory tract epithelial cells. It is able to agglutinate the erythrocytes of different species.

Viral ***N*** is the *sialidase enzyme* that hydrolizes sialic acid in external mucous layer of respiratory tract, thus facilitating viral entry. Also this enzyme is essential for virion release, as it prevents viral particles self-aggregation. Both of the proteins are of strong antigenic activity, causing neutralizing antibodies production.

Certain viral non-structural proteins can be regarded as ***virulence factors*** of virus.

For instance, protein *PB1-F2* affects mytochondria and stimulates apoptosis of infected cells. Protein *NS1* inhibits expression of interferons and other antiviral cytokines. Enzyme *PA-X* with endonuclease activity destroys cellular mRNAs, thereby terminating cell protein synthesis.

Segmented genomic RNA of influenza viruses encoding hemagglutinin and neuraminidase is capable of gene segment re-assemblage, known as ***genetic reassortment***. After possible mixed inoculation of the cell by the viruses of different types (e.g., H1N1 and H3N2) the reassortment can take place, resulting in new influenza virus subtype formation.

Genetic reassortment ensures sharp viral genomic changes, called ***genetic shift***. The shift usually occurs between the genomes of type A human and avian influenza viruses after possible coinfection. This coinfection may occur in the cells of pig host. It leads to the rapid creation of a new virus subtype.

Influenza B and C viruses are deprived of antigenic shift because of little spread of related viruses in animals.

Genetic drift means the minor genetic variations of viral protein-encoding sequences, ensuing from the point mutations in these genes. It leads to gradual viral escape from the action of host specific antibodies.

Virion Resistance

Influenza viruses are relatively stable to cooling and can survive at temperature 0-4^{o}C. They are inactivated at 65°C in 5-10 minutes. These viruses are sensitive to all disinfectants, ether and other organic solvents, UV light and to acidic pH.

Viral Replication Cycle

The replication cycle of influenza virus is rather short (about 6-8 h) being focused in the nucleus of infected cells.

Viral attachment is promoted by interaction of ***hemagglutinin*** with cell-surface ***sialic acid***. For successful binding hemagglutinin should be activated by local proteolysis with host enzymes localized in upper respiratory tract.

Receptor-mediated ***endocytosis*** provides virion internalization. After fusion of viral envelope with the cell membrane the uncoating activates. Acidification of the endosome compartment via ***M2*** protein channells accelerates viral nucleoprotein release into the cytoplasm.

At the first step of viral genomic RNA replication the positive-strand RNA copy is formed. The reaction is catalyzed by virus-encoded RNA polymerase. Positive strand serves as the template for subsequent protein synthesis. All of the genomic segments are sequentially transcribed. Two of newly appeared transcripts undergo splicing.

Hemagglutinin and neuraminidase are produced in the cell endoplasmic reticulum. Then they are transported to the cell membrane for subsequent insertion.

Nucleocapsids are assembled in the host cell nucleus and transferred to the cell membrane. They interact with hemagglutinin and neuraminidase that facilitates budding.

Budding process accomplishes the maturation of nascent viruses and results in massive egress of progeny virions that rapidly infect the neighbouring cells.

Because of short replication cycle, about 1000 virions are multiplied from 1 viral particle in first 8 h, and enormous number of 10^{27} virions arises to the end of the first day of the infection. This ensures the rapid onset and acute course of influenza.

Pathogenesis and Clinical Findings of Infection in Humans

Influenza is the most common and severe human respiratory infection. According to WHO data, seasonal influenza epidemics affect 5-10% of adult population and 20-30% of children. They result in 3-5 mln of severe disease cases annually followed by 250,000-500,000 deaths.

Global pandemics of influenza involve up to 30-40% of human population with millions death cases.

During influenza outbreak the ***source of infection*** is human (patient with the disease). The ***airborne*** (***aerosol***) mechanism of transmission is predominant for influenza virus. Occasionally the disease can be transmitted by direct contact.

Incubation period depends on virus inoculation dose and lasts 1-3 days. Respiratory epithelium is the primary target for viral infection.

Short replication cycle of influenza virus provides the rapid spread of the infection within the epithelium of airways. Neuraminidase degrades the mucous layer of the respiratory tract, promoting viral dissemination. Subsequently the trachea, bronchi, bronchioli, and alveolar epithelial cells become involved into the process. Propagating viruses cause deep cellular destruction with subsequent epithelium desquamation. Also influenza viruses may affect endothelial cells resulting in damage of microcirculation blood vessels.

Influenza is characterized by sudden onset with the headache, dry cough, high fever and muscular aches. Uncomplicated cases of illness are finished within 5-7 days. Children usually display more severe forms of the disease.

Most frequent complication of influenza is ***pneumonia*** development. It can be *primary viral*, *secondary bacterial*, or *combined*. Secondary immunodeficiency, resulting in the defects of secretory IgA synthesis and phagocyte dysfunction, and the impairment of the ciliary clearance elevates the probability of bacterial complications. The combined viral-bacterial pneumonia occurs in 7-10 days of the disease onset and can increase greatly the influenza mortality rate especially in case of *S. aureus* infection.

Rare but severe complication of influenza virus infection is virus-induced encephalitis.

Specific antibodies and cytotoxic cells appear in 12-15 days after the disease onset. After the disease the strong long-active ***subtype-specific immunity*** is formed. Antibodies to hemagglutinin and neuraminidase possess protective activity. Anti-ribonucleoprotein antibodies are type-

specific. They are used for virus typing. Host interferon activity can inhibit influenza virus replication and accelerates the recovery.

Passive immunity is maintained in newborns and infants for 6-8 months after birth.

Pathogenesis of ***avian flu*** caused by H5N1 or H7N9 virus is dramatically different from typical seasonal influenza A disease.

Avian flu viruses, e.g. H5N1 strain, actively circulate in Southeast Asia amongst the aquatic birds (e.g., ducks, which are the natural reservoirs of infection). Periodically they cause devastating epizootics in poultry (primarily, chickens) resulting in destruction of most of their population.

Humans become infected only after the long close ***contact*** with infected birds (e.g., the poultry workers or villagers working with infected flocks) or by ***alimentary*** route. Aerosol transmission or human-to-human transmission of avian influenza is not registered.

Avian ***H5N1*** pathogen is different from human influenza A viruses by the number of substantial traits.

First, it reacts mainly with epithelial cells bearing the sialic receptors with α-2-3-glycosidic bonds; in humans these receptors are somewhere present only in the lower respiratory tract. As the result, airways of humans are poorly susceptible to H5N1 virus. However, if entered into deep airways, H5N1 ultimately causes viral pneumonia with damage of lung tissue.

Second, hemagglutinin of H5N1 is activated by the vast number of non-specific proteases present in all human tissues that predisposes to systemic viral disease.

And third, H5N1 infection triggers a massive production of proinfllammatory cytokines with cell death and tissue damage (systemic inflammatory response syndrome or ***SIRS***).

Hence, if started, H5N1 infection in humans manifests as severe pneumonia, followed by respiratory distress syndrome (***RDS***) and systemic virus infection with endothelial damage and multiorgan failure that often leads to patient's death.

From 1997 to 2016 WHO registered 854 human H5N1 infection cases with 450 deaths (about 50% fatality).

If after the putative reassortment with seasonal influenza A virus H5N1 agent becomes capable of infecting humans by aerosol route with human-to-human transmission, H5N1 infection will pose a tremendous pandemic threat for human population.

Laboratory Diagnosis of Influenza

Laboratory diagnosis of influenza is based on virus isolation and identification.

Nasopharingeal washings and throat swabs are used for the examination. The ***specimens*** should be taken within 3 days after the disease onset.

Rapid methods comprise ***immunofluorescence assay*** of nasal swabs for viral antigen detection and the identification of viral nucleic acid in patient's infected cells by reverse transcription PCR (***RT-PCR***).

For ***viral cultivation*** the 9-10-day-old embryonated chicken eggs and primary monkey kidney cells are used. Inoculation is produced into the media, supplemented with antibiotics to suppress the concomitant bacterial flora, and with trypsin, which activates viral binding to the cell culture.

After three or four days of culture influenza virus is detected by *hemagglutination test* (***virus indication***). If the result is negative, a second passage through the fresh culture is performed.

Virus identification is accomplished by ***hemagglutination inhibition test*** with subtype-specific reference antisera to most prevalent viral strains. Also it can be made by *neutralization of viral cytopathic effect*.

Serological method is used for retrospective influenza infection diagnostics to confirm the identification data of viral type and subtype. Antibodies to H-, N-, or M-proteins and viral nucleoprotein NP are produced in patients with influenza.

For diagnosis confirmation *paired sera tests* are required because the patients can maintain some anti-influenza antibodies level due to previous influenza infections. Hemagglutination inhibition test and ELISA are mainly used. Positive test relies upon the fourfold rise of specific antibody titers.

For identification of a new type of influenza virus the antibodies to viral NP antigens are evaluated. The previously mentioned reactions, as well as complement fixation test and neutralization test are used.

Treatment and Prophylaxis of Influenza. Influenza vaccines

Influenza is extremely contagious. For ***non-specific prophylaxis*** patient’s isolation, air decontamination, rooms ventilating and disinfection significantly prevent the spread of influenza.

Adamantan derivatives (***amantadine*** hydrochloride and ***rimantadine***) were introduced long ago for ***specific antiviral therapy*** of influenza ***type A***. They should be administered only in 1-2 days after the disease onset. The drugs ***block M2*** viral proteins thus preventing the step of virus uncoating. Hence, they are not so effective at the late stages of the disease. Also these medicals can be used for the urgent prophylaxis of influenza during epidemic period. Unfortunately, the rise of resistance of type A viruses to adamantan derivatives is common (e.g., pandemic "swine flu" A/2009/H1N1 agent was totally resistant to these drugs).

Inhibitors of neuraminidase (e.g., ***oseltamivir*** and zanamivir) prevent the release of type A viruses. Overall, the treatment with oseltamivir shortens the total course of influenza A case for about 1 day.

As the result, vaccination is the only actual method to combat influenza epidemics.

For ***specific prophylaxis*** various anti-influenza ***vaccines*** were elaborated.

From the large number of vaccines the most efficient are ***live attenuated*** vaccines, inactivated ***split vaccines*** and ***subunit*** vaccines.

All of them are combined ***polyvaccines*** containing at least 3-4 various antigens of influenza viruses.

Live attenuated vaccines are usually produced by virus culture in embryonated eggs with subsequent virus purification. They confer protective type-specific immunity against the disease. Live vaccines can be administered locally by nasal spray. However, they are contraindicated for the persons suffering from egg protein allergy.

"***Split***" ***vaccines*** include viral particles (virions) decomposed by detergents. Detergent treatment removes lipid envelope from complex viruses, thus exposing inner protein antigens to the immune cells on vaccination. Influenza split vaccines contain viral coats (capsids) with immunogenic outer proteins (namely, hemagglutinin and neuraminidase).

The most advanced products are ***subunit vaccines***. They are composed of different combinations of purified hemagglutinin and neuraminidase glycoproteins (e.g. *Grippol* or *Fluorix* vaccines). These vaccines are safe and strong effective. The latter makes possible the rapid change of vaccine composition according to current epidemic situation.

The individual efficacy of anti-influenza vaccines is 60-90%. Vaccination coverage of population in the range of 70-80% ("***herd***" or ***community immunity***) prevents the emergence of seasonal influenza epidemic.

Nevertheless, post-vaccination immunity lasts only for 6-10 months. Together with possible changes of antigenic composition of epidemic virus it requires the regular design of new versions of influenza vaccines and annual human revaccination.

PARAMYXOVIRUSES

The History of Discovery of Pathogenic Paramyxoviruses

First representative of *Paramyxoviridae* family, measles virus, was detected in 1911 by J. Anderson and J. Goldberger. Then in 1934 C. Johnson and E. Goodpasture isolated the causative agent of epidemic parotitis or mumps – mumps virus. And in 1956 R. Chanock discovered first human parainfluenza virus.

Zoonotic Hendra virus was primarily isolated after infection outbreak in Australia in 1994. Similarly, zoonotic Nipah virus was first isolated in 1998-1999 in Malaysia in the village of Nipah. Later both agents were proved to pertain to paramyxoviruses and placed into the same genus. These viruses are highly pathogenic for humans.

Classification of Paramyxoviruses

The family *Paramyxoviridae* pertain to the order *Mononegavirales*.

Human pathogenic viruses belong to genera *Respirovirus* (with pathogenic species human ***parainfluenza*** virus 1 and 3), *Rubulavirus* (species are human ***parainfluenza*** viruses 2, 4, 5, and ***mumps*** virus), *Morbillivirus*, containing ***measles*** virus species, and genus *Henipavirus* with highly pathogenic species Hendra virus and Nipah virus.

Structure of Paramyxoviruses

All paramyxoviruses contain linear ***single-stranded negative-sense non-segmented RNA***.

These viruses are covered with the external lipid ***envelope*** of ***spherical shape***. The size of virions is rather ***large*** (about 150-300 nm). Sometimes viral particles with the diameter of 400-700 nm can be found.

Viral nucleocapsid displays the ***helical*** symmetry.

Paramyxoviruses contain nucleocapsid proteins bound to viral RNA. Internal protein ***L*** develops ***RNA polymerase*** activity.

Three outer proteins are the structural components of the viral envelope. The ***matrix*** (***M***) ***protein*** supports envelope structure; other two glycoproteins are localized in the viral external spikes. The larger glycoprotein (***HN***) reveals both ***hemagglutinin*** and ***neuraminidase*** activities (in case of the measles virus – H-protein with hemagglutinin activity only). It promotes viral attachment to the susceptible cells. Another ***glycoprotein F*** (or ***fusion protein***) provides membrane fusion and displays hemolytic and cytotoxic activities.

Virion Resistance

The viability of *Paramyxoviridae* virions is rather low. They generally stay infectious for several hours only. These viruses can withstand low-temperature exposure for some time, but heating, the action of ether, organic solvents, disinfectants and UV light readily inactivate them. Measles virus is the most unstable agent – it is inactivated within 30 min at room temperature; hence, the disinfection is not performed in measles.

Viral Replication Cycle

The replication of paramyxoviruses occurs in the ***cytoplasm*** of infected cells.

Their attachment to the host cells is mediated via ***hemagglutinin*** glycoprotein. For measles virus CD46 membrane molecule serves as the cell receptor.

Extracellular proteases of tissues cleave viral ***F protein*** that triggers its fusion activity. It activates the direct fusion of the viral envelope with the cell membrane without endosome formation. Fusion is possible only at neutral pH of the extracellular environment; acidification inhibits virus penetration.

Then envelope-free nucleocapsids are released into the cytoplasm.

After uncoating viral RNA polymerase catalyzes the formation of mRNAs that encode viral proteins.

In addition, viral polymerase produces the positive-strand RNA intermediate (***antigenome***) that serves as the template for synthesis of genomic (–) RNA.

Nucleocapsid assembly is performed in the cytoplasm. Maturating virions migrate towards the cell membrane and interact with HN- and F-proteins. M protein promotes mature particle formation, joining the viral envelope with the nucleocapsid. Finally paramyxoviruses are released from the infected cells by ***budding***.

In case of intracellular protease presence, viral F protein is cleaved and activated progressively, resulting in fusion of the host cell membranes with ***symplast*** and ***syncytia*** formation.

During the replication cycle acidophilic ***cytoplasmic inclusions*** appear within the cells. Measles virus can produce intranuclear inclusions.

Human Infections Caused by Parainfluenza Viruses

Pathogenesis, clinical findings and immunity

According to antigenic structure there are 5 distinct serotypes of parainfluenza viruses corresponding to 5 viral species.

Types 1-4 of parainfluenza viruses are able to cause respiratory diseases in infants and young children (***parainfluenza*** disease). Type 5 is not pathogenic for humans.

The ***source of infection*** is the sick person.

Parainfluenza is transmitted by ***airborne*** mechanism or by direct contact. ***Incubation period*** varies within 2-7 days. Viruses propagate in the respiratory epithelium.

Very often they induce mild upper respiratory tract infections. But in infants types 1 and 2 parainfluenza viruses provoke severe infections, affecting larynx and trachea. Larynx swelling and respiratory obstruction causes the ***croup*** syndrome (*laryngotracheitis* with obstruction and acute respiratory failure). Bronchial hyperreactivity takes part in croup pathogenesis.

Type 3 parainfluenza virus is the predominant agent of *lower respiratory tract inflammation*, resulting in severe ***bronchiolitis*** and ***pneumonia***.

Type 4 of parainfluenza viruses is not so harmful; it causes inapparent or mild respiratory infections.

Maternal antibodies can’t prevent infant’s parainfluenza infection. The ***immunity*** is predominantly mediated by growing secretory antibodies of IgA class that block local viral propagation. Nevertheless, reinfections readily occur.

Laboratory diagnosis

The ***specimens*** are obtained from nasopharingeal washings, nasal and throat swabs, etc.

For accelerated parainfluenza diagnostics various ***rapid methods*** are elaborated. Immunofluorescence assay of nasal swab and ELISA are frequently performed for primary virus detection.

Also ***PCR*** is used for identification of parainfluenza species in clinical specimens.

Virus isolation is made by inoculation of virus-containing material into the primary human cell lines or monkey kidney cell cultures.

Parainfluenza viruses develop indistinct cytopathic effect but reveal the remarkable ***hemadsorption activity*** with guinea pig erythrocytes that is used for viral detection.

Virus type identification is performed by hemadsorption or hemagglutination inhibition tests.

Serological diagnosis is made with paired sera tests. Hemagglutination or hemadsorbtion inhibition tests and ELISA are adopted for clinical use.

Principles of disease treatment and prophylaxis

Potent antiviral agent ***ribavirin*** is used for treatment of severe clinical forms of parainfluenza infection.

Effective parainfluenza vaccines are not designed yet.

Mumps Virus Infection (or Epidemic Parotitis)

Mumps (***epidemic parotitis***) is the specific highly contagious acute virus disease, which primarily affects parotid, sublingual, and submandibular salivary glands with subsequent involvement of other glandular organs and tissues (pancreas, ovaries, or testes), kidneys and central nervous system. Only ***1 serotype*** of virus is known.

Pathogenesis, clinical findings and immunity in mumps

Mumps virus infects humans, predominantly young children. It is ***anthroponotic*** infections, humans are the only ***sources of infection***.

Disease transmission rate in unvaccinated children is near 50-70%. Mumps virus often gives epidemic outbreaks.

The disease is contracted by ***airborne*** route.

Incubation period varies from 2 to 3 weeks.

Primary viral replication is determined in nasal and upper respiratory tract epithelial cells. Then the virus spreads to the salivary glands and further to susceptible tissues and organs.

The most typical symptoms of epidemic parotitis are the enlargement and swelling of the ***parotid gland***.

Systemic ***viral dissemination*** affects different inner organs and central nervous system because of viral ability to propagate within epithelial cells.

Specific pancreatitis and orchitis that may cause sterility are developed. Meningitis and meningoencephalitis are the main complications on central nervous system in epidemic parotitis.

During the disease course the virus is eliminated from the body mainly with saliva and urine.

One-third of patients display inapparent forms of mumps.

Post-infectious specific humoral ***immunity*** is ***lifelong*** and stable due to one antigenic type of virus. Antibodies appear to various viral antigens (HN-, F-proteins, nucleoproteins, etc.) They maintain immune response for many years.

Newborn child is protected by maternal passive immunity within 6 months.

Laboratory diagnosis

Laboratory examination is useful for diagnosis of inapparent forms of mumps and for differential diagnosis.

For express diagnostics immunofluorescence assay with anti-mumps antibodies is used.

For ***virus isolation*** the specimens are obtained from saliva, urine or cerebrospinal fluid. Inoculation is performed into primary monkey kidney cell cultures. Mumps virus displays characteristic ***cytopathic*** effect with cell rounding and giant cell formation. In the cell culture virus is detected by hemadsorption or hemagglutination.

Mumps virus tidentification is performed by hemadsorption or hemagglutination inhibition tests.

Serological diagnosis confirms acute mumps infection by ELISA detection of anti-mumps IgM-class antibodies.

Principles of disease treatment and prophylaxis

Treatment of the disease is solely symptomatic.

High-effective ***attenuated live vaccine*** is used worldwide for mumps ***specific prophylaxis***. Near to total coverage of children with anti-mumps vaccination lowered the disease incidence in most of states to the sporadic

events. Mumps vaccine can be applied as monovaccine, but in most of cases it is administered like ***triple MMR vaccine*** in combination with attenuated measles and rubella viruses. It confers high-grade long lasting immunity.

Measles Virus Infection

Measles is another example of acute respiratory but systemic viral disease. It is caused by specific virus of a ***single serotype*** that pertains to morbillivirus genus.

Measles is extremely contagious – its ***transmission rate*** in unvaccinated persons is almost ***absolute*** – 95-100%. It is solely human ***anthroponotic*** illness.

Measles virus usually gives outbreaks of the disease among children.

Pathogenesis, clinical findings and immunity in measles

Disease transmission is promoted by the ***airborne*** mechanism. ***Incubation period*** lasts from 9 to 18 days.

Virus replicates in respiratory tract epithelium and moves to regional lymphatic nodes (primary ***viremia***). After second propagation in the lymphoid tissue it spreads throughout the body and penetrates into epithelial cells in skin, conjunctiva, endothelium of vessels, respiratory tract, etc.

Virus persists within circulating immune cells that facilitates viremia.

Giant cells appearance and ***symplast*** formation is observed in tissue epithelial cells. Measles virus ***F-protein*** activates intercellular fusion. It permits direct viral spread across the epithelial layer and prevents virions against antibody neutralization.

Prodromal phase lasts several days and it is shown to be highly contagious. Virus is excreted with biological fluids (urine, nasal secretion) and appears in blood. *Filatov's-Koplik's* spots are developed on the buccal epithelium in oral cavity of patients. The spots are the sites of intensive viral replication. This finding is proven to be the *pathognomonic symptom* of measles.

Fever and maculopapular rash are the other main clinical findings of the disease. Eruptive phase lasts about five days. Rash development results from T cell cytotoxic activity, directed against virus-infected capillary endothelial cells. Rash descends from the face to the body and legs and dampens gradually within 1-2 weeks.

The main complications in measles are secondary bacterial pneumonia, otitis, and disorders of CNS (e.g., encephalitis).

The extremely rare but potentially lethal late post-measles complication is *subacute sclerosing panencephalitis*. It may appear in many years after the infection regress because of immune system incapability to eliminate measles agent. The virus persists within CNS, causing gradual damage of neuronal structures with fatal consequences.

Humoral ***immunity*** against measles develops in 10-14 days after the disease onset. It is maintained by circulating antibodies. The ***immunity is lifelong*** and stable that ensues from single antigenic type of virus.

Maternal anti-measles antibodies passively protect newborns for 6 months. They may develop modified mild clinical forms of measles.

Laboratory diagnosis of measles

Laboratory examination is necessary mainly for diagnosis of unclear measles cases.

Material for investigation (nasopharyngeal swabs and blood samples) should be obtained from the last days of prodromal phase till 1-2 day of eruptive period.

For detection of viral RNA reverse transcription PCR (or ***RT-PCR***) may be used.

Virus isolation is performed by specimen inoculation into monkey kidney cells, human amnion cells or HEp-2 cells.

Virus is detected after 7-10 days of culture by characteristic cytopathic effects. Multinucleated giant cells (***symplasts***) and intranuclear and intracytoplasmic ***inclusions*** appear within the infected cells.

Serological diagnosis is carried out with paired sera tests, where fourfold rise in antibody titer is observed. IgM class antibodies are the markers of the ongoing disease. Antibodies titers are evaluated by hemagglutination inhibition test, neutralization reaction and ELISA.

Specific prophylaxis and treatment of measles

Attenuated ***live measles vaccine*** is administered for ***active prophylaxis*** of the disease. Vaccination is considered to confer lifelong immunity with high efficacy. Live measles vaccine is predominantly used for vaccination in combination with attenuated mumps and rubella viral strains generally known as ***measles, mumps, and rubella vaccine***, or ***MMR***.

The ***treatment*** of measles is supportive. If requires, infusion therapy and body detoxication is administered.

Characteristics of Hendra and Nipah Virus Infections

In 1994 in Australia in Hendra suburb of the city of Brisbane the deadly outbreak of zoonotic influenza-like disease was primarily registered in horses that, in turn, infected contact humans with lethal outcomes. The causative agent was identified as a new parainfluenza virus (***Hendra virus***).

Likewise, in 1998-1999 in Malaysia in the village of Nipah a novel zoonotic parainfluenza virus was isolated after the severe animal outbreak that also affected humans with high lethality (***Nipah virus***).

Further outbreaks of Hendra and Nipah infections were repeatedly registered in Australia and Southeast Asia countries (Bangladesh, India, Malaysia, and Singapore).

Zoonotic Hendra and Nipah parainfluenza viruses appeared to be ***highly pathogenic for humans***. The primary sources and natural reservoirs of infections are the fruit bats that spread the viruses to other animals or humans. The diseases are contracted via the direct contact with infected animals or fomites; aerosol or oral transmission is also taken into account.

In humans Hendra and Nipah infections are manifested as systemic viral disorders with encephalitis and severe pneumonia with acute respiratory failure. The lethality of disease outbreaks is very high in the range 40-60%.

Laboratory diagnosis of infections is based on molecular genetic methods (PCR) and serological testing (ELISA).

Human specific prophylaxis and treatment of these infections are not elaborated yet. In Australia the horses are immunized with vaccine against Hendra infection; experimental human Nipah vaccine is under clinical trial now.

PNEUMOVIRUSES

The History of Discovery

J. Morris and R. Chanock isolated the primary representative of pneumoviruses – respiratory syncytial (or RS) virus in USA in 1956.

First human metapneumovirus was isolated by A. Osterhaus and colleagues in 2001 after the infection outbreak in Netherlands.

For the longest time pneumoviruses were regarded as the members of *Paramyxoviridae* family. Only the latest ICTV release of virus taxonomy and nomenclature published in 2015 ultimately demarcated them and placed pneumoviruses into separate family.

Classification of Pneumoviruses

The family *Pneumoviridae* pertain to the order *Mononegavirales*.

There are two genera in the family: *Orthopneumovirus* with human pathogenic species ***respiratory syncytial virus*** or ***RS virus***, and *Metapneumovirus* with pathogenic species human metapneumovirus.

Structure and Reproduction of Pneumoviruses

The structure of pneumoviruses resembles to a certain extent the composition of paramyxoviruses but with the number of distinct traits.

All pneumoviruses contain linear ***single-stranded negative-sense non-segmented RNA***. They are also covered with the external lipid ***envelope*** of ***spherical shape***.

Viral nucleocapsid displays the ***helical*** symmetry. nucleocapsid proteins are tightly bound to viral RNA. The virus carries ***RNA polymerase*** enzyme.

The large glycoprotein receptor of pneumoviruses is devoid of hemagglutinin and neuraminidase activity, and it is called ***G protein***. The fusion ***F protein*** of respiratory syncytial virus lacks hemolysin activity.

Reproduction of pneumoviruses is generally similar to paramyxoviruses. RS virus binds to glycosaminoglycan receptors of respiratory epithelium, e.g. in lower respiratory tract. The infected cells produce characteristic cytopathic effect with ***syncytium*** formation mediated by viral F proteins.

Infections, Caused by Pneumoviruses – Pathogenesis, Clinical Findings and Immunity

Human ***respiratory syncytial virus*** is the prime cause of ***lower respiratory tract*** diseases in newborns and infants under the age of 2 years.

Respiratory syncytial infection is transmitted by ***airborne*** (aerosol) route. ***Incubation period*** varies for 2-8 days. Virus multiplies in nasopharyngeal epithelial cells with subsequent descending to lower respiratory tract (bronchioli, alveoli).

Cell-mediated immunity limits the viral spread, thus viremia doesn't occur.

The adults usually display the symptoms of common cold infection, but in patients above 50 years with chronic obstructive pulmonary disease (COPD) or immunocompromised the course of infection might be severe.

RS virus in infants often results in serious damage of lower airways, primarily affecting bronchioli and alveoli. The life-threatening complication of RSV infection is severe ***bronchiolitis*** with acute bronchial obstruction and ***pneumonia***.

Virus disintegrates respiratory epithelium with syncytium formation; the damage is intensified by immune cell-mediated reactions. Excessive cytokine production promotes virus-induced inflammation. However, RS virus blocks interferon synthesis by host epithelial cells.

As RS infection provokes bronchial hyperreactivity, it predisposes to the development of bronchial asthma.

Usually the patients recover completely after the RSV infection. Bronchiolitis and pneumonia in infants can cause lethal outcome without adequate therapy.

Specific humoral and cellular ***immunity*** is unstable and of moderate grade. Mucous tissue IgAs are assumed to be responsible for protection. Nevertheless, the repeated exacerbations of RSV infection occur easily. Maternal antibodies defend the newborns against RSV infection during several months after birth.

The first outbreak of respiratory infection caused by human ***metapneumovirus*** was registered in Netherlands, where the virus was primarily isolated in 2001. Since that time, metapneumoviral respiratory infections became regularly diagnosed in many countries. The disease affects the susceptible persons of all ages.

Pathogenesis and clinical manifestations of these disorders are generally similar to RSV infection – human metapneumovirus damages lower respiratory tract resulting in bronchiolitis and pneumonia.

Laboratory Diagnosis of Respiratory Syncytial Infection

For ***rapid diagnostics immunofluorescence*** assay or ***ELISA*** are commonly used for virus detection in nasopharyngeal secretions. Various kinds of ***RT-PCR*** tests are available for viral nucleic acid identification in clinical specimens.

Nasopharyngeal washings, nasal and throat swabs are taken for ***virus isolation***.

The material is inoculated into continuous cell lines HeLa and HEp-2. Respiratory syncytial virus grows slowly, thus the cytopathic effects appear only at 10 day of culture – RSV induces giant cell and syncytium formation. Immunofluorescence test provides rapid virus identification in the cell culture.

Viral indication in clinical specimen is the strong argument for RSV infection, because healthy persons are not able to carry respiratory syncytial virus.

Serological diagnosis is helpful only for epidemiological investigations.

Treatment and Prophylaxis of RSV Infection

Ribavirin is recommended for antiviral therapy of RSV-mediated disorders. For urgent post-exposure prophylaxis of RSV infection in contact infants humanized antiviral monoclonal antibody (mAb "palivizumab") is administered. Palivizumab is directed against F protein of RS virus. The efficacy of mAb prophylaxis is about 50-55%.

Efficient anti-RSV vaccine is not available now. Different kinds of RSV vaccines are under the current laboratory and clinical trials.

RUBELLA VIRUS

The History of Rubella Virus Discovery

Rubella virus (***RV***) is the causative agent of the same name ***rubella*** disease. The earliest description of rubella goes back to the 1700s, when the clinical manifestations of this illness were described by two German physicians, de Bergan in 1752 and Orlow in 1758. At this time it was

considered to be a derivative of measles, thus the disease became known as German measles. The illness was renamed "***rubella***" (*Lat.* – reddish) in 1866 by H. Veale.

Y. Hiro and S. Tasaka in 1938 established the viral etiology of the disease. In 1941 N. Gregg reported the devastating teratogenic effects of the virus. And finally, the isolation of rubella virus culture was elaborated by P.D. Parkman and T.H. Weller with F.A. Neva in 1962.

Classification of Virus

Rubella virus is placed into *Togaviridae* family. It is classified as the single species of the genus *Rubivirus*.

Humans are the only known natural hosts for RV.

Structure of Rubella Virus

Rubella virion is a ***spherical*** particle about 60 nm in diameter. Virion ***envelope*** is composed of host-derived lipid bilayer with embedded spikes made up of ***E1*** and ***E2*** glycoproteins.

Icosahedral internal capsid, or core, made of ***C*** proteins holds viral genome. Genome is composed of non-segmented ***single-stranded RNA*** of ***positive*** polarity.

Viral genome encodes several enzymes, including ***replicase*** (RNA-dependent RNA polymerase), protease and helicase.

Virus possesses core protein antigen and surface external glycoprotein antigens E1 and E2, which develop hemagglutinating activity and can cause the synthesis of neutralizing antibodies. Only ***one serotype*** of virus exists.

Virion Resistance

The viability of rubella viruses beyond the human body is rather low. They stay infectious outside only for about 1 day. These viruses gradually lose infectivity under freezing; heating at 50-56^{o}C kills them in 5-20 minutes. They are readily inactivated by ether, organic solvents, disinfectants and UV light. Also they are sensitive to pH fluctuations losing the stability at pH levels less than 6.8 and above 8.0.

Viral Replication Cycle

RV enters susceptible cells via ***endosomal*** uptake. Viral E1 and E2 glycoproteins under endosome acidification stimulate the fusion of the viral envelope to the endosomal membrane. Uncoating occurs within the endosome, allowing next release of viral genomic RNA. Low pH of the endosome promotes not only virion envelope fusion but also triggers uncoating of the capsid protein.

Virus-modified cytoplasmic endosome-lysosomal structures (termed as ***replication complexes***) are the sites of RV replication.

Viral genomic (+) RNA is translated on ribosomes creating early viral proteins (i.e., enzymes replicase, protease and helicase). Initially single ***polyprotein*** chain is formed that is further processed into final proteins by proteases.

Replicase enzyme catalyzes the synthesis of genomic positive RNA through the stage of double-stranded RNA intermediate, containing both positive and negative RNA strands. Positive RNAs serve as templates for next synthesis of non-structural and capsid viral proteins.

Replication complexes contain small vesicles, where viral morphogenesis takes place.

After assembly virus is released out of the cell by ***budding***. It is not followed by cell lysis.

Overall, the duration of replication cycle of rubella virus is long and covers 36-46 hours.

Pathogenesis, Clinical Findings and Immunity in Rubella

Rubella affects ***unvaccinated*** individuals. The infection is transmitted via ***airborne*** route. Transmission rate for non-vaccinated exceeds 80-90%.

Vertical "mother-to-child" transmission results in ***congenital rubella syndrome***.

Rubella is ***anthroponotic disease*** – the ***source of infection*** is solely human.

The upper respiratory tract and nasopharyngeal lymphoid tissue are the first sites of viral replication. Next the virus migrates to regional lymph nodes.

Incubation period of the disease lasts 2-3 weeks.

The clinical manifestations of RV infection ***in adults*** are generally mild, and many infections are ***asymptomatic***.

The first clinical sign of rubella is usually the appearance of a macropapular rash. Other symptoms typically include low-grade fever, lymphadenopathy, sore throat, etc. Lymphadenopathy is typical, involving the posterior cervical and occipital nodes. Rubella can cause complications with joint involvement such as transitory arthritis.

However, the main threat for public health is the ***teratogenicity of rubella virus***.

Maternal infection early in pregnancy results in ***congenital rubella syndrome*** (***CRS***) in infants. The time the infection affects women during gestation can influence CRS outcome. The earlier in gestation the maternal infection occurs, the more severe is the damage to the fetus. Maternal infection during the first 8 weeks inevitably results in fetus disease. In this condition up to 100% of infected fetuses develop congenital defects.

The virus penetrates all fetal tissues. RV is supposed to affect mitochondria, interfering cell respiration, and can cause apoptosis of infected cells.

The risk of fetal infection and the severity of congenital abnormalities decreases after the first trimester; after 17 weeks gestation, the risk of developing any defects is low.

The clinical manifestations of CRS vary significantly. The deafness is the most common. Other clinical features include cardiac disease, mental retardation, and ocular impairments such as cataracts and glaucoma. Non-inflammatory necrosis is observed in affected organs due to viral action.

After disease lifelong humoral IgG-mediated ***immunity*** arises. Maternal antibodies protect the newborns against rubella within 4-6 months after birth.

Laboratory Diagnosis of Rubella

A leading test for ***detection of viral nucleic acids*** directly in clinical ***specimens*** (nasopharyngeal washings, blood, urine, fetal autopsy materials) is ***RT-PCR***.

Nasopharyngeal washings, nasal and throat swabs are taken for ***virus isolation***.

The virus is inoculated into monkey (e.g. Vero cells) or rabbit cell lines. Virus produces slight cytopathic effect. Immunofluorescence assay or ELISA can be used for virus detection in the cell cultures.

Serological diagnosis is confirmed by ELISA test. It might be carried out with paired sera, where fourfold elevation of antibody levels is observed.

Rubella-specific ***IgM antibodies*** are detected with ELISA in the single specimen.

Principles of Disease Treatment and Prophylaxis

Rubella itself is a mild self-limited disease that doesn't need specific treatment.

A ***live rubella vaccine*** is commonly used for ***specific prophylaxis*** of rubella. It confers high-grade lifelong immunity with few side effects. Vaccination resulted in a substantial decrease in the number of cases of rubella and congenital rubella syndrome. Usually rubella vaccine is a constituent of multivalent respiratory disease vaccine, e.g. ***MMR*** (measles, mumps and rubella attenuated live vaccine).

Vaccination coverage of population above 95% prevents the cases of rubella infection making conditions for virus elimination.

CORONAVIRUSES

The History of Discovery

First human coronavirus was isolated by D. Tyrrell and M.L. Bynoe from patient with acute rhinitis in 1965.

It was generally assumed earlier that coronaviruses cause only mild short-lasting respiratory or enteric diseases with complete patient's recovery. But in 2002 the epidemics of new severe disease emerged in Southeast Asia, especially in China and Vietnam. The disorder primarily affected respiratory tract, resulting in fatal outcome near 10% of total disease cases. The disease was termed as "severe acute respiratory syndrome", or ***SARS***. In 2003 it was firmly established that the causative agent of SARS is the previously unknown new coronavirus (C. Urbani and coworkers, 2003). The virus was named as ***SARS coronavirus*** (SARS CoV) and placed into separate group of coronaviruses. Investigating SARS, Italian doctor Carlo Urbani contracted the infection and died.

Finally, in 2012 the severe disorder similar to SARS emerged in Saudi Arabia. Its agent, a novel coronavirus, was isolated by Egyptian virologist doctor Ali Mohamed Zaki.

By analogy with SARS, the illness was termed as ***MERS*** (*Middle East respiratory syndrome*), and its causative agent was entitled as ***MERS coronavirus*** (MERS CoV).

Classification of Coronaviruses

The family *Coronaviridae* pertain to the order *Nidovirales*.

The family comprises two subfamilies – *Coronavirinae* and *Torovirinae*.

Coronavirinae subfamily contains 4 genera each of several species. To date 6 species of coronaviruses are registered, which are definitely pathogenic for humans.

SARS and MERS coronavirus species pertain to genus *Betacoronavirus*.

According to serological and genetic properties, 3 groups of coronaviruses are known; groups 1 and 2 harbor mammal pathogens; group 3 includes avian viruses.

Human pathogens are present in both 1st and 2nd group; SARS and MERS coronaviruses pertain to group 2.

Structure and Properties of Coronaviruses

Coronaviruses carry ***single-stranded positive RNA***. It is the largest viral RNA genome known.

Coronaviruses produce the middle-size or large ***spherical*** particles with external lipoprotein ***envelope***. Viral nucleocapsid is of ***helical*** symmetry. Matrix intermediate layer scaffolds the outer coat of coronaviruses. Glycoprotein ***spikes*** surround viral envelope resembling ***sun crown***.

Coronaviruses contain various proteins of nucleocapsid, matrix and outer coat that determine complex viral antigenic structure. Viruses of animal and human origin possess the number of common and specific antigenic determinants.

External glycoprotein spikes are responsible for viral absorption and penetration into the host cells. They display hemagglutinating activity.

The most studied is ***SARS coronavirus***.

On the base of genomic analysis and electron microscopy data the main properties of SARS viruses were determined.

SARS virus has 4 major structural proteins – inner nucleocapsid ***N*** protein, membrane ***M*** and envelope ***E*** proteins, and ***S*** protein of receptor spikes.

Viral genome also encodes the number of viral enzymes including *RNA-dependent RNA polymerase* (***replicase***), helicase and proteases.

Multiple non-structural ***accessory proteins*** play a role of ***virulence factors*** for SARS virus. They stimulate apoptosis of infected cells, inhibit the expression of interferons of I type, grossly activate proinflammatory cytokines, and cause deep disorders of blood coagulation and fibrinolysis.

If cultured, coronaviruses are poorly adapted to laboratory animals and cell lines. Some strains can propagate in green monkey kidney cell cultures or Vero cells, and in suckling mice.

Virion Resistance

Coronaviruses are rather sensitive to external influences; ether, detergents and other disinfectants as well as heating above 56°C readily inactivate them.

SARS CoV is relatively stable in comparison with other coronaviruses. It maintains viability in feces and urine for 2-4 days at room temperature. It easily withstands cooling being stable at least for 3 weeks at low temperature range.

Heating at 56^{o}C kills virus within 15 min. As other coronaviruses, it is sensitive to all conventional disinfectants.

Replication Cycle of SARS Coronavirus

SARS virus enters epithelial cells of upper respiratory tract via binding of *spike **S*** proteins to specific cell membrane receptor *angiotensin-converting enzyme 2* (***ACE 2***). S-glycoprotein mediates absorption and subsequent fusion of virus particles with susceptible cells.

Uncoating is followed by translation of viral proteins from viral (+) RNA. Initial single *polyprotein* chain is produced that is eventually cut into mature proteins by viral protease.

Newly formed viral replicase enzyme activates genomic RNA replication. Viral genome is transcribed through intermediate minus RNA

strand that serves as the template for final positive sense RNA synthesis.

Viruses multiply in the ***cytoplasm*** of infected cells. After virion assemblage the viral particles are released out of the cells by ***budding*** that is promoted by *M*, *E* and *N proteins*.

The death of infected epithelial cells is caused by direct viral cytopathic effects as well as by immune cell lysis. The latter results from immune reaction against viral antigens expressed upon infected cells

Characteristics of Common Respiratory Coronaviral Infections

Coronaviral infections are transmitted by ***airborne*** route. Known before 2002 coronaviruses cause up to 30% of all common ***acute respiratory infections*** and some gastrointestinal disorders.

In adults respiratory infections are usually asymptomatic, or produce rhinitis and some other modest catarrhal manifestations. Secondary viremia leads to the infection of gastrointestinal tract resulting in diarrhea. Typically the infection course ceases in 5-7 days.

The ***immunity*** is mostly humoral and type-specific. Virus-neutralizing antibodies confer the resistance to reinfection with the same type virus.

Laboratory Diagnosis, Prophylaxis and Treatment of Coronaviral Infections

Cell culturing is rarely applied due to poor viral propagation in the cell lines.

For ***express analysis*** of nasal swabs or nasopharingeal washings *immunofluorescence assay* for viral antigens and *PCR* for identification of viral nucleic acid are elaborated.

Serological testing is commonly used in routine laboratory diagnosis of coronaviral infections. Paired sera tests (e.g. *indirect hemagglutination assay*, or *ELISA*) are most preferable; they determine the 4-fold rise of specific antibodies in patient's serum.

Vaccination is not available for prophylaxis of coronaviral diseases. The infection needs only supportive treatment.

Epidemiology, Pathogenesis and Clinical Findings of SARS

At the end of 2002 the first cases of severe "atypical" pneumonia of unknown etiology have emerged in Guangdong Province of China. The

infection rapidly spread throughout the Southeast Asia involving humans in Hong Kong, Vietnam, and then in Canada and Europe. As the infection demonstrated high mortality rate (near to 10%) and great communicability by airborne route, the World Health Organization (WHO) declared a global alert for this disease, designated as "severe acute respiratory syndrome". The epidemic was curbed during 2003; it resulted in more than 8000 infection cases with 774 lethal outcomes.

Overall, SARS was regarded as the first threatening epidemic of XXI century.

The majority of investigators suppose SARS virus to originate from wild animal coronavirus via the chain of occasional mutations. In 2003 Chinese researchers found the virus causing severe acute respiratory syndrome in masked palm civet – a feliform mammal species eaten as a delicacy in China. This agent is regarded now as the animal virus, apparently related with SARS. Thus, the particular animal species is possible source for initial SARS infection.

Also it has been established that the long-term natural reservoirs for SARS virus are fruit bats.

Nevertheless, the ***sources of infection*** in verified SARS cases were ***sick humans***. The disease is contracted predominantly via ***airborne*** route. ***Human-to-human transmission is common***. Household contact and contacts in health-care settings are also ascertained as the important routes of transmission. Oral transmission of the disease is possible as well.

The healthcare workers and household members, who cared for patients with the disease, are regarded as the groups of highest risk of SARS acquisition. Special attention is to be paid on travellers returned from areas of infection outbreaks, especially from Southeast Asia.

Incubation period lasts from 1 to 7-10 days.

Specific cell membrane receptor for SARS virus *angiotensin-converting enzyme 2* is present in high density on airways epithelium, endotheliocytes and enterocytes, which are the major primary targets for SARS infection.

The disease has sharp onset with fever and myalgia. The virus affects respiratory system causing severe lung damage. Multiple ***virulent accessory proteins*** of SARS virus cause cell death and provoke systemic inflammatory response. Hematogenous dissemination of virus involves gastrointestinal tract that results in diarrhea.

The fever may decline in a few days but on the 2-3 week of the illness many patients develop a secondary wave of fever with progressing respiratory failure. Since the viral replication slows down near the second

week of the illness, the life-threatening disease progression largely ensues from the activation of host autoimmune reactions. About 20% of patients manifest respiratory distress syndrome (***RDS***) with alveolar pulmonary edema that can cause the lethal outcome.

Prognosis of SARS is deeply serious. Nevertheless, introduction of advanced methods of the disease treatmen substantially reduced the number of fatal outcomes.

The infection confers strong high-grade ***immunity*** maintained by specific antiviral antibodies. They render high virus-neutralizing activity.

Special Characteristics of MERS Infection Outbreaks

As mentioned above, first outbreak of *Middle East respiratory syndrome* (***MERS***), followed by successful isolation of its agent ***MERS coronavirus*** (***MERS CoV***) was registered in 2012 in Saudi Arabia.

According to WHO data, from 2012 to 2016 MERS infection spreaded from its intial focus in Middle East to Southeast Asia countries; the infection was also registered in Europe, United States, and African countries. On August, 2016 the total number of MERS cases was equal to 1791. Case-fatality ratio of MERS is extremely high – about 35%.

MERS virus and MERS disease to some extent are similar with SARS. Nevertheless, MERS infection demonstrates several distinct traits.

MERS CoV is ***zoonotic*** virus supposed to originate from local animals (mainly, Arabian camels). Humans become infected by alimentary route and after close contact with sick animals or humans (hospital-acquired infection).

MERS is manifested as severe systemic inflammatory disease that initially damages airways with development of respiratory distress syndrome and acute respiratory failure; this is followed by intestinal disorders and renal disfunction that may result in acute renal failure.

Current MERS epidemic is not completely controlled yet, as the separate cases of infection repeatedly occur nowadays, e.g. in Saudi Arabia. Nevertheless, MERS infection is not regarded as global epidemic threat primarily because of low human-to-human transmission rate.

Laboratory Diagnosis of SARS

Laboratory diagnosis of SARS infection is verified by ***PCR*** and *serological reactions*, e.g. by ***ELISA test***.

For PCR virus-containing ***specimens*** are taken from nasopharingeal washings, feces, and occasionally from urine.

Laboratory handling of SARS clinical specimens should be performed in special biosafety facilities (***BSL-2*** – biosafety level 2).

The diagnosis of SARS is confirmed by ***PCR*** in case of at least two positive repeated tests.

For ***serological diagnosis ELISA*** test is elaborated. In patients the reaction is carried out with paired sera tests, where fourfold rise in antibody levels ultimately validates the infection. Healthy persons are negative for specific antibodies to SARS CoV.

Virus culturing is produced in most advanced specialized laboratories using various cell lines (e.g., Vero cells). Virus isolation is confirmed by PCR.

Principles of SARS Prophylaxis and Treatment

Global alert from SARS infection requires strict measures to prevent international spread of the disease. It needs professional and public awareness, heightened surveillance with rapid case validation, patient isolation and management. Additional measures should be directed to control travellers departing from the areas of disease outbreak.

International affiliation of scientific laboratories and institutions organized by WHO as Global Outbreak Alert and Response Network, joins collaborative forces to respond to SARS, MERS, or any other emerging infection threat.

Vaccine for SARS prevention is not yet elaborated; nevertheless, the availability of complete genomic sequence of SARS agent makes possible to design effective vaccines and antiviral agents.

Antiviral drug ***ribavirin*** is commonly used for ***treatment*** of SARS infection. To suppress immunopathological reactions aggravating the disease course steroid hormones (e.g. prednisolone or hydrocortisone) can be administered. The treatment of respiratory distress syndrome presumes the support of vital body functions including fluid resuscitation and artificial lung ventilation if required.

ADENOVIRUSES

The History of Adenovirus Discovery

These viruses were discovered by W. Rowe and coworkers in 1953. Primary investigation of adenoviruses revealed their ability to persist and develop the cytopathic effect in adenoids and tonsils, so they were named ***adenoviruses***.

Classification of Adenoviruses

Adenoviruses pertain to the family *Adenoviridae* that encompasses 5 distinct genera. Currently known 7 human pathogenic species (human adenviruses A-G) are included into genus *Mastadenovirus*, which contains adenoviruses, affecting mammals.

From 100 adenoviral human serotypes known, above 50 serotypes are pathogenic for humans. They are clustered into several groups.

Structure of Adenoviruses

Adenoviruses are ***DNA***-containing ***naked viruses***. Viral DNA is the ***linear double-stranded*** molecule, which is attached to the protein at the end of genome. DNA is packed into the core of the virion.

Virus size varies in the range of 70-100 nm. The virions are of ***icosahedral symmetry***.

Adenoviral capsid is composed of 252 capsomeres, where 12 capsomers are ***pentons***, (polyhedrons, based on pentagon structure) and other 240 are ***hexons*** (units, based on hexagons). Spike-like capsid structures, known as ***fibers***, are connected with pentons.

Fibers develop hemagglutinating activity, and pentons display cytopathic effect in the cell cultures.

Pentons, hexons and fibers are the major antigens of adenoviruses, containing group- and type-specific epitopes on their surface. Fibers carry type-specific epitopes that are used for virus serotyping.

Virion Resistance

Virions of adenoviruses are highly stable in the environment. Protein capsid protects them from unfavorable influences. They stay viable in water for weeks, on dry inanimate surface – up to 3 months

Adenoviruses easily resist freezing. Heating at a temperature of 56^{o}C inactivates them in 30 minutes; 60^{o}C – in less than 10 minutes. They are insusceptible to ether and detergents, but destroyed by chlorine-containing disinfectants and formaldehyde.

Viral Replication Cycle

Adenoviruses propagate within epithelial cells. Viral attachment is promoted by fibers. They bind to the specific cell receptors. Viral penetration is facilitated by ***penton*** interaction with cellular ***integrins***. Adenoviruses are transported rapidly from the endosomes towards the cell ***nucleus***, where the uncoating is finished.

About 20 early nonstructural proteins are synthesized, providing DNA replication. After DNA replication, the late proteins are translated.

Capsomeres are self-assembled in the nucleus. The duration of adenovirus replication cycle is about 24 hours.

Pathogenesis and Clinical Findings of Adenoviral Diseases in Humans

Adenoviruses affect human epithelial cells, causing cell aggregation and enlargement usually without lysis. Moreover, they suppress immune defensive factors. For instance, adenoviruses inhibit interferon action by blocking interferon-induced genes transcription. Viral proteins also inhibit cytotoxic activity of TNF-alpha. Finally, they slow down HLA-Ag I class expression on the membranes of infected cell, preventing cell-mediated cytolysis.

Adenoviruses are highly versatile in their pathogenicity. Various human serotypes affect respiratory and gastrointestinal tracts, eyes, and urinary bladder. They propagate in the epithelial cells and spread to regional lymph nodes, where they persist. The leading sites of adenoviral persistence are adenoids and tonsils. Also adenoviruses replicate in the epithelium of intestinal tract.

Adenoviral infections are spread by ***airborne*** and ***fecal-oral*** transmission. Infants and young children are the most susceptible to the infection.

Infectious dose of adenoviruses is ***very low*** – dozens or even several viral particles.

The ***sources of infections*** are sick or convalescent individuals. Shedding of adenoviruses after the infection may be long (for 3-6 weeks).

About 50% of cases of human adenoviral infections are mild or asymptomatic. Nevertheless, they provoke a great number of acute and chronic human diseases. Among them are:

– acute febrile pharyngitis;
– pharyngoconjunctival fever;
– acute respiratory diseases in children and adults;
– adenoviral pneumonia;
– adenoviral gastroenteritis;
– eye infections;
– acute hemorrhagic cystitis;
– urethritis and cervicitis.

Acute febrile pharyngitis resembles other similar viral respiratory infections.

Pharyngoconjunctival fever manifests by the symptoms both of pharyngitis and conjunctivitis. It is often caused by adenoviruses of serotypes 3 and 7; outbreaks of pharyngoconjunctival fever are called "swimming pool conjunctivitis".

Adenoviruses of types 3 and 7 as well as the limited number of other serotypes cause ***adenoviral pneumonia***. It comprises about 10% of all pneumonia cases affecting children. Lethality of ***adenoviral pneumonia*** in early postnatal period may exceed 5%.

Adenoviruses of types ***40*** and ***41*** mainly affect gasrointestinal tract, being responsible for about 10% of cases of ***viral gastroenteritis*** in infants.

Eye adenoviral infections include ***follicular conjunctivitis*** and ***epidemic keratoconjunctivitis***. The latter is predominantly caused by 8, 19, and 37 viral types.

Viruses of types 11 and 21 provoke ***acute hemorrhagic cystitis*** in children, while type 37 causes ***urethritis*** and ***cervicitis***.

Systemic adenoviral infection in infants leads to serious complications affecting parenchymatous organs (hepatitis, nephritis), heart (myocarditis), CNS (meningoencephalitis).

After most adenoviral diseases strong long-lasting ***immunity*** is developed. It is maintained by type-specific neutralizing antibodies. Group-specific antibodies can't prevent the relapse of infection.

Laboratory Diagnosis of Adenoviral Infections

The clinical ***specimens*** are obtained from the throat swab or conjunctivall swab, from stool or urine.

Nasal epithelial cells of patients can be examined directly by *immunofluorescence test* to reveal viral antigens in the infected cells.

Virus isolation is carried out in different human cell lines – HeLa, HEp-2, etc.

The indication is performed by cytopathic effect with rounding, enlargement, and grape-like clustering of impaired cells.

Identification is made by viral cytopathic effect neutralization, or by inhibition of hemagglutination test.

Molecular-based assays are broadly used for viral DNA identification. DNA hybridization and ***PCR*** can identify various groups of the adenoviruses.

Fecal specimens can be directly examined by electron microscopy or by latex agglutination and ELISA tests for adenovirus presence.

Serological testing is used to detect the growth of antiviral antibodies during the infection course. Latex agglutination and ELISA are usually performed in paired sera test, and the fourfold elevation of specific antibodies is regarded as positive test. Viral neutralization and hemagglutination inhibition tests can be applied as well.

Principles of Disease Prophylaxis

Non-specific prophylaxis of adenoviral infections is achieved by maintaining of asepsis conditions, sterilization of medical instruments, chlorination of swimming pools and wastewaters, etc.

For specific prophylaxis formaldehyde-treated vaccine had the limited use in past. By now, the vaccine is not available. The novel types of adenovirus-specific vaccines are under the research, including type-specific live vaccines.

The treatment is supportive. Specific antiviral agents are not elaborated.

Chapter 16

HUMAN ENTERIC VIRUSES: ROTAVIRUSES, PICORNAVIRUSES

REOVIRUSES AND ROTAVIRUSES

The History of Discovery

First agents (later designated as reoviruses) were isolated in 1951 by US virologist W. Stanley from the feces of Australian child with fever, pneumonia and gastroenteritis. Further in 1959 A. Sabin termed them ***reo****viruses* (*reo* means ***r****espiratory* ***e****nteric* ***o****rphan* viruses) as they were isolated from human airways and gastrointesinal tract without evident relations with certain human pathology.

These viruses were placed into separate family *Reoviridae*.

New members of this family – rotaviruses – were isolated by R. Bishop in 1973. They are common etiological agents of diarrheal diseases in infants.

Classification of Viruses

Reoviridae family currently comprises two subfamilies and 15 genera.

The genus ***Rotavirus*** pertains to subfamily *Sedoreovirinae*. This genus embraces 8 viral species (*Rotavirus A-H*).

Human infections are caused mainly by 3 species *Rotavirus A*, *B* and *C*, but more than 90% of infection cases are produced by ***Rotavirus A*** species members.

Depending on variations of external capsid proteins *VP7* (G-protein) и *VP4* (P-protein) and their genes, all rotaviruses are divided into multiple serotypes and genotypes.

Other representatives of reoviruses, e.g. from genera *Coltivirus* and *Orbivirus*, are the transmissive zoonotic agents; in certain conditions they may also afflict humans.

Structure of Reoviruses and Rotaviruses

Virion size is 70-100 nm in diameter. Viruses possess ***two capsid shells***, made of proteins; each capsid is of ***icosahedral*** symmetry.

Inner composition of viral particle has a triple-layered structure. Virion is devoid of lipid envelope (***naked viruses***).

Reoviral genome consists of ***double-stranded RNA***, composed of 10-12 discrete ***segments***. Rotaviruses contain 11 genome segments.

Virion of ***rotaviruses*** looks like wheel (Lat. *rota* – wheel).

Mature capsid of rotaviruses has 6 structural (*VP1-VP7* except *VP5*) and 6 non-structural proteins (*NSP1-NSP6*).

External capsid is composed of proteins ***VP4*** (or G-protein) and ***VP7*** (or P-protein). They play a role of viral ***receptors***, binding to polysaccharides of enterocytes.

Protein *VP1* is RNA-dependent RNA polymerase.

Certain non-structural proteins act as rotavirus ***virulence factors***. The most active is viral ***enterotoxin NSP4*** with multiple deleterious activities.

NSP4 activates chloride secretion by enterocytes and increases intracellular Ca concentration; the latter results in cytoskeleton impairment, damage of tight junctions between enterocytes and stimulation of proinflammatory cytokine secretion. Taken together, this leads to ***secretory*** and ***inflammatory diarrhea***.

Protein ***VP3*** inhibits interferon synthesis.

Segmented genomic RNA of rotaviruses encoding superficial VP4 and VP7 proteins is capable of gene segment ***recombination***, known as ***genetic reassortment*** or ***genetic shift***.

After possible coinfection of target cells by rotaviruses of different genotypes the reassortment can take place – it results in formation of recombinant virions with a new genome and new combination of VP4 and VP7 proteins within external coat. Recombination between human and animal rotaviruses is also possible.

Genetic drift means the minor genetic variations of viral VP4- and VP7-encoding sequences, ensuing from the point mutations in their genes. This exerts viral evasion from the host immune response.

Despite high genetic variability, human infections are caused by only limited number of genotypes and serotypes of rotaviruses.

Virion Resistance

Reoviruses and rotaviruses are stable in the environment – outside the host they maintain viability for several months. Virions withstand pH changes in the range of 3.0-9.0; therefore, they are more resistant in gastric juice. Nevertheless, rotaviruses are inactivated by heating at 50^{o}C for 30 minutes. They are resistant to lipid solvents, but susceptible to 95% ethanol, phenol, chlorine, and glutaraldehyde. Limited treatment with proteolytic enzymes increases infectivity.

Rotavirus Replication Cycle

Viral particles attach via capsid receptors ***VP4*** and ***VP7*** to sialic acid residues and oligosaccharides of enterocyte membrane receptors.

Viruses enter the cell by ***endocytosis*** that is followed by removal of external capsid. Reproduction of rotaviruses occurs in ***cytoplasm*** of infected cells.

Core-associated viral RNA polymerase is activated. It transcribes mRNA molecules from the minus strand of each genome segment. Released mRNAs are translated into viral proteins and serve as templates for synthesis of negative-sense strands with subsequent double-stranded genome formation.

At the same time viral polypeptides self-assemble to form the inner and outer capsid shells.

Intensive reproduction and egress of progeny virions results in ***lysis*** of infected cells.

Rotaviruses are the ***fastidious*** agents to ***culture***. They don't propagate in embryonated eggs or experimental animals. Nevertheless, group A human rotaviruses are adapted to certain cell lines. They grow if pre-treated with trypsin that facilitates uncoating. Maturation of virions is incomplete and slow. Reoviruses produce the inclusion bodies in the cytoplasm of infected cells.

Pathogenesis and Clinical Findings of Rotaviral Diseases

Rotaviruses are the ***leading causative agents of gastroenteritis*** that predominantly affects infants and children before 5 years.

Acute rotaviral gastroenteritis with diarrhea is a most common disease that creates the significant burden on public health worldwide but especially in developing countries. According to WHO data, it accounts for at least 500,000 children lethal cases annually.

Rotaviruses are ubiquitous. By age of 3 years, 90% of children have serum antibodies to one or more types of rotaviruses.

The ***source of infection*** is sick human.

Rotaviral infection is transmitted via ***fecal-oral*** mechanism with contact, foodborne and waterborne routs. The infant becomes infected predominantly by the ***direct contact*** with virus-contaminated fomites.

Infectious dose of virus is very low – about 100 viral particles.

Incubation period is short (1-2 days).

Rotaviruses pass through the stomach being resistant to acidic gastric juice. They infect duodenal and intestinal epithelium by binding to intestinal villi. The viruses multiply in the cytoplasm of enterocytes and impair electrolyte trans-membrane transport. One of the rotavirus-encoded proteins, ***NSP4***, is a viral ***enterotoxin***, which triggers diarrhea activating intestinal chloride secretion. Also it causes direct damage of enterocytes and stimulates intestinal inflammation. Damaged cells may slough into the lumen of the intestine and release large quantities of virus, which sheds with feces.

Typical symptoms of rotaviral infection include fever, abdominal pain, diarrhea and vomiting, resulting in severe dehydration.

In infants and children, severe loss of electrolytes and fluids may be fatal without compensatory infusion therapy. Patients with milder cases have symptoms for 3-8 days and then recover completely.

Viral excretion usually lasts 2-12 days. Normal function of intestine is restored only in 3-8 weeks after the disease.

Local ***immunity*** factors, such as ***secretory IgAs***, may be important in protection against rotavirus infection.

Laboratory Diagnosis of Rotaviral Infection

As all acute viral diarrheas are similar in clinical manifestations, the diagnosis of rotaviral infection rests on laboratory testing of infection origin.

Rotaviruses are fastidious for culture; hence, laboratory diagnosis is based on ***virus detection*** in ***stool specimens*** taken early in the disease course.

Molecular ***genetic tests*** (e.g., ***RT-PCR***) play a pivotal role in rapid and precise identification of rotaviral nucleic acids in clinical specimen.

Virus in stool can be detected also by immune electron microscopy, or by ELISA.

Serological diagnosis evaluates fourfold growth of antibodies by ELISA or latex-agglutination tests.

Principles of Treatment and Prophylaxis of Rotavirus Infections

Treatment of gastroenteritis is supportive, directed to correction of water and electrolyte loss. It includes fluids resuscitation and restoration of electrolyte balance either intravenously or orally.

For ***specific prophylaxis*** of rotavirus infection two efficient ***live attenuated vaccines*** are applied (RV1 and RV5). They are successfully used in more than 80 countries. Mass vaccination of children substantially improves epidemiological situation with rotavirus infection worldwide.

PICORNAVIRUSES

The History of Picornavirus Discovery

The investigation of picornaviruses has commenced as far back as in 1908-1909, when K. Landsteiner and E. Popper demonstrated the viral nature of poliomyelitis. However, poliomyelitis virus was isolated only in 1949 by J. Enders.

In 1948 G. Dalldorf and G. Sickles discovered a new enterovirus during infection outbreak in the town of Coxsackie (USA); hence, it was designated as Coxsackie virus.

First representatives of the numerous group of ECHO viruses were isolated and studied by M Ramos-Alvarez and A. Sabin in 1953-1956.

Classification of Picornaviruses

Picornaviruses pertain to the same name ***order*** *Picornavirales* and ***family*** *Picornaviridae*. This family comprises more than 30 viral genera. The genera of primary medical relevance are *Enterovirus*, *Parechovirus*, and *Hepatovirus*.

Most of pathogenic human viruses belong to ***genus Enterovirus***. It embraces 12 species – 9 enteroviral ***species*** *Enterovirus A-J* (without *I*) and 3 rhinoviral ***species*** *Rhinovirus A, B, C*.

By their antigenic variations enteroviruses are additionally divided into serogroups encompassing more than 100 ***serotypes***.

Species *Enterovirus A* contains serotypes of ***group A Coxsackieviruses***.

Species *Enterovirus B* includes ***group B Coxsackieviruses*** and more than 30 serotypes of ***ECHO-viruses***.

Species *Enterovirus C* comprises ***polioviruses*** of **1**, **2**, **3** serotypes, and the rest of serotypes of ***group A Coxsackieviruses***.

In addition, *Enterovirus A, B,* and *C* species harbor many enteroviral serotypes that are not included into certain viral serogroup.

Rhinovirus A, ***B***, and ***C*** species represent more than 150 serotypes.

Parechovirus genus (species – ***parechoviruses A*** and ***B***) has 14 serotypes; some of them may affect humans.

Hepatovirus genus has single serotype ("*serotype 72"*).

Besides above mentioned agents, in rare cases the members of other picornaviral genera *Cardiovirus* and *Kobuvirus* may cause human pathology.

Finally, the representatives of genus *Aphthovirus* cause ***foot-and-mouth disease*** – severe highly contagious epizootic disorder affecting domestic and wild even-toed ungulates – e.g., cattle, sheep or swine.

Structure and Properties of Picornaviruses

Picornaviruses are ***single stranded positive-sense RNA***-containing ***naked viruses***.

Virus size is very ***small*** (28-30 nm). Viral nucleocapsid possesses ***icosahedral symmetry***. It is composed of 60 capsomers. Four structural proteins *VP1-VP4* of enteroviruses are folded into capsomer unit. Capsid proteins of many picornaviruses possess hemagglutinin activity.

VP4 is tightly bound to viral RNA. Also viral nucleocapsid has small inner *Vpg* protein.

Slightly distinct parechovirus capsomers are composed of 3 viral proteins. These proteins develop major antigenic activity of enteroviruses.

Virus culture of polioviruses is performed in various cell lines. They don't propagate in experimental animals or embryonated eggs.

Coxackieviruses are highly pathogenic for newborn ("suckling") mice.

Virion Resistance

Picornaviridae members are highly resistant viruses. They can withstand acidic pH, UV irradiation, long-term drying, etc. Viruses can survive in water for about 100 days, in milk for 90 days, in feces and in sewage for several months.

Enteroviruses are sensitive to chlorine-containing disinfectants. Heating at 56° inactivates them in 30 min.

Viral Replication Cycle

Virus attachment is performed via specific receptors of host cell membrane. Polioviruses interact with their specific membrane receptor ***CD155***, whereas many other enteroviruses and all rhinoviruses bind to *intercellular adhesion molecule* ***ICAM-1*** (or ***CD54***). Specific binding induces viral conformational change with subsequent viral penetration by ***endocytosis***. Acidification of endosome facilitates viral uncoating and RNA release. All cycle of virus propagation takes place in ***cytoplasm*** of infected cells.

Viral uncoating is followed by genomic (+) RNA translation, which results in large single ***polyprotein*** synthesis. It is rapidly cleaved into several structural and non-structural proteins, including RNA-dependent RNA polymerase (***replicase***) and viral protease. Subsequent RNA replication occurs through the stage of negative-sense RNA intermediate, which serves as template for viral genome synthesis.

Viral self-assemblage starts from genomic RNA pack into capsid proteins. After complete maturation the progeny viruses are released from the host cells resulting in cell disintegration and ***lysis***.

Picornavirus replication cycle takes about 5-10 hours.

Enteroviral Diseases in Humans

Poliovirus infection – pathogenesis, clinical findings and immunity in poliomyelitis

Structural properties of polioviruses are typical for enteroviruses. Polioviruses don’t contain hemagglutinin.

Poliomyelitis is an infectious disease that is manifested as severe ***central nervous system disorder*** that may result in ***flaccid paralysis***.

Poliomyelitis affects solely ***humans***, which are the only known reservoir of infection (***anthroponotic*** disease).

Infection is transmitted via ***fecal-oral*** mechanism (waterborne and alimentary routes).

Incubation period ranges 1-2 weeks.

Primary viral replication is performed within tonsils, and then in Peyer's patches and intestine epithelial cells. The virus is excreted with stools prior to the disease onset.

After propagation in the lymphoid and epithelial tissues it undergoes blood dissemination and reaches CNS along axons of peripheral nerves.

Infection of CNS by polioviruses can result in deep destruction of spinal cord neurons (predominantly, motor neurons of anterior horns) and brain neurons with subsequent irreversible paralysis. Polioviruses don't multiply in muscles *in vivo*. The impairment of innervation of striated muscles is secondary to the destruction of neurons.

Despite active CNS involvement, most poliomyelitis cases are subclinical (*inapparent*) or mild.

Clinical forms of poliomyelitis infection are the follows:

– ***abortive poliomyelitis*** is the most common manifestation of the disease; the patient has only fever, malaise, headache, nausea, and recovers in a few days;

– ***nonparalytic poliomyelitis*** that may result in aseptic viral meningitis; it is characterized by stiffness, back and neck pain and meningeal symptoms together with above manifestations;

– ***paralytic poliomyelitis*** arises in about 1% of total disease cases; it results in flaccid paralysis due to motor neuron damage; maximal recovery may occur within 6 months after the infection, but residual paralysis lasts much longer.

Nowadays the cases of poliovirus infection are predominantly registered as ***vaccine-associated poliomyelitis*** (***VAP***) after the immunization with live poliovaccine.

Virus-neutralizing antibodies elevate soon after the exposure to infection. However, polioviruses located in brain and spinal cord are not influenced by high titers of blood antibodies.

Post-infectious ***immunity*** is high-grade and long-lasting, but only ***type-specific***.

Passive immunity is transferred from mother to newborn; nevertheless, the levels of maternal antibodies gradually decline during first 5-6 months after birth

Laboratory diagnosis of poliomyelitis

Throat swabs taken soon after the onset of illness, stool samples or rectal swabs are used as ***specimens*** for laboratory examination.

Viral cultivation is made in WHO recommended genetically modified murine cell culture that expresses CD155 or in human rhabdomyosarcoma RD cultures.

Cytopathic effects appear in 5-7 days on inoculation. Isolated virus is identified by neutralization tests with specific antisera that distinguish 3 basic serotypes of polioviruses.

For laboratory confirmation of cases of ***vaccine-associated poliomyelitis*** (or ***VAP***) molecular ***genetic tests*** are applied (***PCR*** and viral genome ***sequencing***), as they are able to identify mutant viral strains within the same polio serotype.

Serological testing is used to evaluate the growth of antibody titers during the course of the disease.

Principles of treatment and prophylaxis of poliomyelitis

In case of poliovirus infection only ***symptomatic treatment*** is available. Administration of human donor's immunoglobuln containing antiviral antibodies may foster patient's recovery.

For ***specific prophylaxis*** both live- and killed-virus vaccines are commonly used. ***Inactivated polio vaccine*** (***IPV*** or *Salk's vaccine*) is prepared from the virus grown in cell cultures. Killed vaccine induces humoral antibodies, but doesn't stimulate local intestinal immunity.

Oral trivalent vaccine contains live attenuated virus (*Sabin's vaccine*) grown in cultures. This polio vaccine multiplies in human intestinal epithelium. Live vaccine treatment produces not only IgM and IgG antibodies in the blood, but also secretory IgA antibodies in the intestine. As the result, live vaccine confers both systemic and local immunity.

Oral vaccine is administered at least thrice because of 3 distinct serotypes of polioviruses.

Extremely rare cases of vaccine-associated paralytic poliomyelitis (***paralytic VAP***) may occur in vaccinated with live poliovaccine resulted from possible vaccine strain mutations.

The applications of genetic engineering create the opportunities for the development of a live poliovirus recombinant vaccine that can't mutate to

virulent strain. Nevertheless, current strategy of polio vaccination in many countries (e.g., in Belarus) relies on administration of inactivated vaccine only to preclude virus human circulation and to escape putative VAP cases.

The Global Polio Eradication Initiative governed by the World Health Organization (WHO) from 1988 strives for eradication of poliovirus on the Earth as it was done for smallpox virus.

Coxackieviral Infections

Special features of Coxsackie viruses

Coxackieviruses are similar with other enteroviruses. They are divided into two groups, ***A*** and ***B*** with multiple serotypes spread among various enteroviral species. Most of Coxackie viruses possess hemagglutinin.

Pathogenesis, clinical findings and immunity

The ***incubation period*** of coxsackieviral infection ranges from 2 to 9 days.

Coxsackieviruses produce a great variety of disorders in humans:

– herpangina or vesicular pharyngitis;
– aseptic meningitis and meningoencephalitis;
– respiratory febrile diseases;
– pleurodynia or epidemic myalgia;
– hand, foot and mouth disease or vesicular stomatitis with exanthem;
– acute hemorrhagic conjunctivitis;
– generalized disease of infants;
– various clinical forms of myocarditis and pericarditis;
– hepatitis, pancreatitis.

It is generally ascertained that ***group B*** coxsackieviruses attack presumably inner organs, whereas ***group A*** viruses affect central and peripheral nervous system and muscular system.

Various diseases can be spread among humans via ***fecal-oral*** or ***airborne*** transmission.

Virus has been recovered from the blood in the early stages of infection in humans. It is also found in the throat for a few starting days of infection and in the stools for up to 5-6 weeks.

Coxsackie herpangina is a severe febrile pharyngitis. It is caused by certain group A viruses. There is an abrupt onset of fever and sore throat with discrete vesicles on the soft palate, pharynx, tonsils, or tongue. The disorder is self-limited and most common in young children.

Hand, ***foot*** and ***mouth*** disease also known as ***vesicular stomatitis with exanthem*** may affect persons of all ages but predominantly young children before 4-5 years. It is caused by group A Coxsackie viruses and by enterovirus serotype 71.

This self-limited but contagious ailment is manifested by damage of skin and mucosa with ulcerations and eruptions of small vesicles in oral cavity, upon hands and feet.

Pleurodynia, or ***epidemic myalgia***, or *Bornholm disease* is provoked by group B viruses. It is followed by fever, chest and abdominal pain. The disease is also self-limited and recovery is complete; nevertheless, the relapses are common.

Coxsackie ***myocarditis*** is a serious disease with acute inflammation of myocardium and pericardium (***pericarditis***). It can occur in adults as well as in children. Infection can be fatal in neonates. Coxsackie B virus infections are supposed to trigger host autoimmune response that leads to ***cardiomyopathies***.

Aseptic meningitis is similar with same disorders of picornavirus origin. Fever, malaise, headache, nausea, and abdominal pain are common clinical findings. The muscle weakness occurs, but the patients usually recover completely.

Generalized disease of infants is followed by total coxsackie viremia, affecting baby's heart, liver, and brain. The disease is caused by group B coxsackieviruses and has a serious prognosis.

Seroepidemiological data links ***type 1 (insulin-dependent) diabetes mellitus*** with coxsackie B viruses.

Immunity in Coxsackie infections is mediated by type-specific neutralizing antibodies that appear early and persist for years. Antibodies are transferred passively from mother to fetus, protecting newborns for about 6 months.

Laboratory diagnosis of Coxsackie infections

Specimens are taken from throat washings during the first several days of infection and from feces during the first few weeks. In cases of aseptic meningitis, the viruses can be recovered from cerebrospinal fluid as well as from intestine.

Viral cultivation is performed by sample inoculation into cell cultures and by infection of suckling mice. In cell cultures a cytopathic effect evolves within 5-14 days. In suckling mice, signs of the disease appear usually within 3-8 days with group A strains and 5-14 days with group B

strains. The virus is identified by neutralization reaction or by hemagglutination inhibition test.

Serological testing is performed in paired sera tests, where fourfold rise in antibody titer is observed. ELISA and hemagglutination inhibition tests are available.

Principles of prophylaxis and treatment of infections

There are no vaccines or antiviral drugs currently applied for prevention or treatment of diseases caused by coxsackieviruses.

Infections, Caused by ECHO Viruses

Special features of ECHO viruses

Echoviruses (abbreviation of *enteric cytopathogenic human orphan viruses*) are grouped together as they infect human enteric tract and can be recovered from humans by inoculation into certain cell lines. More than 30 serotypes are known, but not all of them affect humans.

Echoviruses display typical traits of enteroviruses. Several echovirus strains possess hemagglutinin.

Pathogenesis, clinical findings and immunity

Echoviruses can cause different ***alimentary infections*** (e.g. gastroenteritis), ***aseptic meningitis***, encephalitis, acute ***respiratory*** viral infections, ***generalized viral disease of newborns*** and other viral disorders usually with non-specific findings.

Parechoviruses of serotype 1 cause literally the same disorders like group ECHO viruses.

Pathogenesis is similar to other enteroviral diseases.

Echoviruses must be regarded as causative agents in outbreaks of aseptic meningitis and in summer seasonal epidemics of viral disorders with fever and rash, especially in young children.

Nowadays echoviruses are the major cause of ***aseptic meningitis*** in children. With the potential elimination of poliomyelitis in developed countries, the pathology of CNS associated with echoviruses is prevalent.

Immunity maintained by neutralizing antibodies is long-term and type-specific.

Laboratory diagnosis

It is difficult to diagnose an echoviral infection on clinical grounds.

Isolation of virus is possible from throat swabs, stools, rectal swabs, and, in case of aseptic meningitis, from cerebrospinal fluid.

For clinical specimen testing various ***rapid methods*** are employed. Immunofluorescence assay of throat swab and ELISA are used for primary virus detection.

Viral cultivation is available in cell cultures (e.g. primary monkey kidney cells). If a viral agent is isolated, it is tested against the wide number of antisera against enteroviruses. Determination of viral type present is made by immunofluorescence or neutralization tests. Infection with two or more enteroviruses may occur simultaneously.

As some echoviruses carry hemagglutinin, hemagglutination inhibition test is suitable in these cases for viral identification.

For ***rapid identification*** of various enteroviral strains reverse transcription PCR (***RT-PCR***) is widely used.

Serological diagnosis is impractical because of the many different viral types.

Principles of infection treatment and prophylaxis

Administration of immune globulin for infants with suspected enterovirus infections is of limited value. There are no antiviral drugs or vaccines available for the treatment or prevention of any echoviral disease.

Other pathogenic enteroviruses

Certain other serotypes of enteroviruses can cause human disorders.

Enterovirus 68 accounts for ***bronchiolitis*** or ***pneumonia***, being isolated from respiratory tract of children.

Enterovirus 70 is the major cause of acute hemorrhagic conjunctivitis.

Enterovirus 71 was revealed in patients with meningitis, encephalitis, and paralysis that resembles poliomyelitis. It can produce serious central nervous system disorders that may be fatal. Enterovirus 71 strains can cause the meningoencephalitis that affects brain stem neurons. Also type 71 infection can result in pulmonary edema and hemorrhages with lethal outcome.

Chapter 17
HEPATOTROPIC VIRUSES

Viral hepatitis is a ***systemic disease*** primarily ***affecting the liver***.

The cases of viral hepatitis in children and adults are caused by one of the following hepatotropic viruses:

hepatitis A virus (***HAV***), the etiologic agent of viral hepatitis type A (previously termed as infectious hepatitis);

hepatitis B virus (***HBV***) that causes viral hepatitis B (known in past as "serum hepatitis");

hepatitis C virus (***HCV***) – the agent of the same name hepatitis C;

hepatitis D virus (***delta virus*** or ***HDV***) – the agent of hepatitis D or delta hepatitis;

hepatitis E virus (***HEV***), the agent of enterically transmitted hepatitis E.

All above listed pathogens afflict humans only.

Hepatotropic viruses produce acute or chronic inflammation of the liver, resulting in a clinical disease manifested by fever, gastrointestinal disorders, and symptoms of liver damage, e.g. jaundice.

Some outsider viruses like GBV-C, TTV or SEN were accounted for a long time as putative causative agents of other hypothetical viral hepatites (e.g., hepatitis G or F).

However, now it is evident that in spite of wide distribution of some pathogens in human population (like GBV-C pegivirus or TTV anellovirus) there are no firmly established associations between these viruses and liver disorders in humans.

HEPATITIS A VIRUS

The History of Virus Discovery

Viral etiology of epidemic jaundice was not wide accepted by physicians until the middle of 20^{th} century, in spite of G. Findlay, J. Dunlop, and H. Brown data, published in 1931, where they proposed a virus as the most probable etiological agent of previously known "catarrhal jaundice". This clinical syndrome was ascertained later as hepatitis A disease.

Hepatitis A virus was discovered in 1973 by S.M. Feinstone and coworkers from the data of immune electron microscopy of patient's feces.

Classification

Hepatitis A virus (***HAV***) pertains to *Picornaviridae* family.

Despite HAV shares some major characteristics with other genera of picornaviruses, it is sufficiently different and classified as the only species in the separate genus *Hepatovirus*. There are naturally occurring strains that infect nonhuman primates (three genotypes) as well as four genotypes that comprise human-infectious viruses. Only ***one serotype*** is known.

Structure of Virus

HAV is a 27-32 nm ***spherical*** particle with ***icosahedral*** symmetry, containing a linear ***single-stranded (+) RNA*** genome.

Intact virions have nucleocapsid with RNA, covalently linked VPg protein, and a capsid of the coat proteins VP1-VP3.

Virion resistance

HAV demonstrates high virion stability. It withstand treatment with ether and acids (e.g., pH 1.0 for 2 hours), as well as heating (60°C for 10 h). Outside the host it stays viable for months.

The virus is destroyed by UV irradiation, autoclaving, boiling in water for 5 minutes, by treatment with formaldehyde or chlorine-containing disinfectants.

Viral Replication Cycle

HAV propagation assumes to be ***exclusive to hepatocytes*** and gastrointestinal epithelial cells. Viral replication is similar with other picornaviruses. Uncoating is followed by genomic (+) RNA translation with formation of structural and non-structural proteins, including RNA polymerase. It replicates viral RNA genome via a negative strand intermediate. Viral proteins are further produced from the genomic positive RNA strand. Virus particles appear in bile and blood, being released mainly across the apical hepatocyte membrane into the biliary canaliculus and across the basolateral membrane into the bloodstream. The mechanism of viral release and secretion is not known yet but it is usually not followed by hepatocyte necrosis.

Pathogenesis, Clinical Findings and Immunity of Hepatitis A

The ***source of infection*** is sick person. The disease affects predominantly children or young adults.

Hepatitis A ***transmission*** occurs via ***fecal-oral route*** (waterborne and foodborne infection) and by direct contact.

Incubation period lasts in the range 10-50 days (average – about 1 month).

Primary replication of virus occurs in enterocytes and in regional lymph nodes. At the end of incubation period HAV enters the blood resulting in *viremia*. Viruses enter hepatocytes from systemic bloodstream or directly via the portal vein.

Enterohepatic cycling of HAV is observed. After replication within hepatocytes the viruses are secreted into bile with subsequent excretion with feces. Otherwise, they undergo next reabsorbtion in the gut. The latter results in repeat viral uptake by hepatic cells. The enterohepatic cycle continues until specific antibodies eliminate virus.

Disease onset is followed by fever, jaundice, bilirubin and aminotransferase elevation.

In young children, acute HAV infection is very often asymptomatic. In contrast, elder children and adults demonstrate a range of clinical manifestations from mild infection to potentially fatal fulminant hepatic disease.

Nevertheless, complete recovery occurs in majority of hepatitis A cases; ***chronicity is not observed***.

HAV infection stimulates both humoral and cellular ***immunity***.

IgM, IgG, and IgA antibodies directed against HAV proteins are induced and can usually be detected by the onset of clinical symptoms. They neutralize virus activity, facilitating recovery.

By contrast, cell-mediated immunity provokes immune-mediated hepatocyte injury, aggravating hepatic inflammation.

Post-infectious IgG-mediated humoral immunity provides ***lifelong protection*** against HAV infection.

Laboratory Diagnosis of HAV Infection

Liver biopsy permits a tissue diagnosis of hepatitis A. Tests for abnormal liver function, such as serum alanine aminotransferase (ALT) and bilirubin supplement the clinical, pathological, and epidemiological findings.

HAV can be detected in various clinical specimens (the liver biopsy, stool, bile, or blood) by various immunoassays (immunofluorescence test, ELISA), nucleic acid hybridization assays, or PCR.

Peak levels of virus are detected in the stool about 1-2 weeks prior to the first liver enzyme abnormalities.

Virus particles are detected by immune electron microscopy in fecal extracts of hepatitis A patients. Virus appears early in the disease and disappears within 2 weeks following the onset of jaundice.

Serological diagnosis is most suitable for clinical use.

Anti-HAV antibodies appear in the IgM fraction with peak near 2 weeks after elevation of liver enzymes. Anti-HAV IgG antibodies follow the onset of disease and persist for decades.

Thus, the detection of IgM anti-HAV antibodies in blood confirms the diagnosis of hepatitis A with specifity of 99%. ELISA is the method of choice for measuring HAV antibodies.

Principles of Hepatitis A Treatment and Prophylaxis

Subclinical and mild cases of viral hepatitis A don't require treatment. ***Treatment*** of manifested cases is supportive, specific antiviral drugs are not available.

For passive prophylaxis of exposed persons human immunoglobulin can be administered.

For ***specific prophylaxis*** various cultural ***inactivated*** vaccines are commonly used. Children are immunized twice at 18 and 24 months.

Vaccine-induced antibodies are long-living and persist in the least for 20 years. Vaccination confers long-term protection against HAV; as the result, future eradication of this infection is possible now.

HEPATITIS E VIRUS

History of Discovery

In 1981 Soviet virologist M.S. Balayan made an experiment of self-infection, ingesting the infectious material collected from 9 soldiers with hepatitis of unclear origin. At 37^{th} day from the infection time point he

manifested typical symptoms of acute viral hepatitis. Laboratory testing revealed no markers of already known viral hepatites A or B. Nevertheless, immune electron microscopy of fecal specimens demonstrated the presence of novel hepatitis virus. This way hepatitis E virus (or HEV) was discovered.

Meanwhile, some previous epidemics of fecal-orally transmitted hepatites were later proven to be hepatitis E disease. For instance, it was documented for New Delhi outbreak of 1955 (India), where 29,000 cases of hepatitis with jaundice were registered after sewage contamination of the drinking water supply.

Classification

Hepatitis E virus (***HEV***) pertains to *Hepeviridae* family, genus *Orthohepevirus*, and species *Orthohepevirus A*. This virus has 4 genotypes.

Genotypes 1 and 2 are the solely human pathogens; genotypes 3 and 4 are isolated from humans and various animal species (swine, boars, rabbits, deers, etc.)

Other members of genus *Orthohepevirus* infect only animals (zoonotic viruses).

Structure of HEV

HEV is a 27-34 nm ***spherical*** particle with ***icosahedral*** symmetry, containing a linear ***single-stranded (+) RNA*** genome. It is ***naked*** virus without lipid envelope.

Three genetic regions of HEV (open reading frames, ORFs) encode viral proteins. One ORF codes for viral ***enzymes*** (RNA-polymerase, protease and helicase); two others encode viral ***capsid protein*** – major HEV antigen, and ***phosphoprotein***, participating in viral infection.

Only ***1 serotype*** of virus is determined.

Virion Resistance

Generally HEV is less stable than hepatitis A virus. It is inactivated by heating at 60°C for 15-30 minutes. Also HEV is destroyed by chlorine-containing disinfectants and formaldehyde.

Nonetheless, the stability of HEV virions is quite enough for fecal-oral transmission.

Viral Replication Cycle

Virus attachment is performed via ***capsid protein*** binding to hepatic heparan sulfate and heat shock protein receptors. Viruses enter hepatocytes by ***endocytosis***. All cycle of virus propagation takes place in ***cytoplasm*** of infected cells.

Viral uncoating is followed by genomic (+) RNA translation, which results in *early proteins* synthesis (e.g., viral enzymes). Newly formed viral RNA polymerase (***replicase***) catalyzes viral (+) RNA replication through the stage of negative-sense RNA intermediate, which serves as template for viral genome synthesis. Later the structural proteins of HEV are translated.

Viral self-assemblage depends on ***capsid*** proteins; viral ***phosphoprotein*** facilitates virion translocation and egress. After complete maturation the progeny viruses are released from hepatocytes by ***budding***.

Pathogenesis, Clinical Findings and Immunity of Hepatitis E

Hepatitis E demonstrates 2 basic clinical forms, depending on viral genotype.

HEV of ***1-2 genotypes*** cause ***mass outbreaks*** of the disease in developing countries of Southeast Asia, Africa, and Latin America. This is mainly related with poor drinking water supply and sanitary control burdened with common deficiency of water resources. Less intensive outbreaks of infection may be registered in many countries all over the world.

HEV of ***3*** and ***4 genotypes*** cause ***sporadic*** cases of hepatitis E worldwide. Last 15 years a steady growth of this infection is observed in many developed world regions (United States, European countries, Russia, Japan, New Zealand, etc.) Now it is generally ascertained that this variant of the disease is typical ***zoonotic*** infection transmitted by alimentary route.

Hence, hepatitis E caused by HEV of ***1-2 genotypes*** is the disease with ***waterborne*** transmission. The ***sources of infection*** are humans.

And hepatitis E caused by HEV of ***3-4 genotypes*** is zoonotic infection with ***alimentary*** (***foodborne***) transmission primarily via ingestion of contaminated meat. The ***sources of infection*** are various animal species (mostly swine or boars).

Incubation period lasts from 2 to 6 weeks.

The details of pathogenesis of hepatitis E are not completely elucidated. HEV enters the liver via portal vein and replicates in hepatocytes. Damage of liver cells is predominantly mediated by ***cellular autoimmune response*** directed against virus-infected host cells.

The virus actively discharges from the body with feces especially in early stages of infection.

Epidemic hepatitis E caused by HEV of ***1-2 genotypes*** predominantly affects young individuals. Only 40% of patients display jaundice; the course of infection is usually moderate or mild. Lethality of epidemic outbreaks doesn't exceed 1%. Complete recovery occurs in most of cases; ***chronicity is not observed***.

However, in case of infection of pregnant women at third trimester of pregnancy, severe and fulminant forms of the disease can develop with expected high fatality rate (20-30% and even more). The mechamisms of this clinical condition are not well-elucidated yet.

Sporadic ***alimentary*** hepatitis E caused by HEV of ***3-4 genotypes*** affects mainly elder persons with substantial comorbidity or under immunosuppression. The disease generally demonstrates more active course with jaundice in 75% of patients. If the patient had preliminary chronic liver disease (chronic hepatitis or cirrhosis), the fatality of HEV infection may be high (up to 70% in 1 year from infection onset). Chronic forms of hepatitis E can be observed in these patients.

HEV infection confers both cellular and humoral post-infectious ***immunity***. The protection maintains for several years. However, the levels of specific antibodies gradually decline, and HEV reinfection becomes possible.

Laboratory Diagnosis

Serological testing is primarily used for HEV laboratory diagnosis. Anti-HEV IgM antibodies are determined in patient's sera by ELISA indicating acute disease.

Molecular genetic tests (e.g. ***reverse transcriptase PCR***) detect HEV RNA in patient's feces. PCR and genetic sequencing identify genotypes of hepatitis E viruses.

Prophylaxis and Treatment of HEV Infection

In most of cases viral hepatitis E needs only supportive symptomatic ***treatment***. Ribavirin and interferon are used in immunocompromised patients.

For ***specific prophylaxis*** several HEV recombinant vaccines were developed, based on viral capsid protein. One of them is registered in China for mass vaccination.

HEPATITIS B VIRUS

The History of Virus Discove*ry*

In the first half of XX century it has become evident that at least two viral agents are responsible for so-called "infectious jaundice". As the result, F.O. MacCallum and D. J. Bauer proposed in 1947 the designation of hepatitis B for "serum-transmitted" hepatitis and hepatitis A for the disease transmitted via fecal-oral route.

In 1967 B.S. Blumberg discovered a specific antigen in blood of an Australian aborigine that was later associated with acute hepatitis B and led to the development of specific tests for identification of HBV infection.

Finally, viral etiology of hepatitis B was firmly established by D.S. Dane in 1968-1970 by electron microscopy with detection of HBV virions (referred to as ***Dane particles***). Dane particles were shown to react with antiserum to Australian antigen. Next study found this antigen to be the surface component of HBV virion, thereby termed as hepatitis B surface antigen (or ***HBsAg***).

Classification of Virus

Hepatitis B virus (***HBV***) pertains to *Hepadnaviridae* family, genus *Orthohepadnavirus*.

This genus also harbors other viral species that infect animals (e.g., bats, woodchucks, squirrels, etc.)

Hepatitis B virus cause acute and chronic viral hepatitis, often progressing to permanent carrier states, liver cirrhosis and hepatocellular carcinoma.

Viral Structure

HBV is a 42 nm ***spherical*** particle with ***icosahedral symmetry***, ***enveloped*** with external ***protein shell***.

HBV genome contains unique ***circular partly double-stranded DNA***. Minus strand of DNA is almost a complete circle and carries overlapping genes that encode both structural proteins (*pre-S, surface,* and *core proteins*) and replicative proteins (*polymerase with reverse transcriptase function* and *X-protein*). The plus strand of DNA is shorter and variable in length. The gap in DNA must be completed at beginning of replication cycle.

Partially different 8 genotypes of HBV are known to date (A to H).

HBV contains 4 major antigens – ***HBs***, ***HBc***, ***HBe*** and ***HBx***.

Viral envelope is predominantly composed of HBsAg and some lipids. External shell surrounds a 27-nm inner ***nucleocapsid core*** that contains HbcAg.

HbsAg particles exist in several forms – S (small), M (middle) and L (large). The latter two variants are composed of HbsAg and ***pre-S2 protein component*** or ***pre-S1 component***.

It is suggested that the pre-S proteins play an important role in the attachment of HBV to hepatocytes. In addition, pre-S2 attaches to polymerized human serum albumin.

Core HBcAg is processed intracellularly to produce ***HBeAg***, which appears in bloodstream.

HBx protein is a small transcriptional transactivator, regulating viral transcription. It is suspected to participate in emergence of HBV-induced primary hepatocellular carcinoma.

Also HBV genome encodes large ***polymerase*** (***P***) ***protein*** that develops ***DNA polymerase***, ***reverse transcriptase***, and ***RNase*** activities.

Virion Resistance

HBV virion is a stable particle. The dried virus remains viable for at least 1 week. HBV can be stored at -20°C for over 20 years; it is resistant to repeated freezing and thawing.

HBV virion (but not HBsAg) is sensitive to high temperatures being inactivated at 100°C in 1 minute. Incubation at 60°C prolongs viral survival to 10 hours.

HBsAg remains stable at pH 2.4 for up to 6 hours, but HBV infectivity is lost.

HBV is relatively sensitive to a number of disinfectants such as halides, glutaraldehyde and formaldehyde, 95% ethanol, phenol. For instance, sodium hypochlorite destroys HBV antigenicity within several minutes.

HBV Replication Cycle

HBV replication begins with binding of the virus to hepatocyte membrane with subsequent penetration and shedding of external shell.

Virus core is transported to the nucleus, where the relaxed circular DNA is converted to a *covalently closed circular DNA* (***cccDNA***), which acts as the template for all viral RNA synthesis.

DNA transcription results in ***pregenome RNA*** synthesis. Pregenomic RNA serves as a template for reverse transcription resulting in a negative strand DNA copy. The polymerase starts to synthesize positive DNA strand, but the process is not completed. Sometimes DNA becomes capable of integrating with cellular genome, but it usually doesn't occur during the normal course of replication.

Viral RNAs are translated on ribosomes yielding viral structural proteins.

Viral cores become encapsidated with newly synthesized HbcAg, acquire HBsAg-containing envelopes in cytoplasm, and may exit the cell. Alternatively, cores may be reimported into the nucleus and initiate another round of replication in the same cell.

Pathogenesis, Clinical Findings and Immunity of Hepatitis B

HBV infection is grossly spread worldwide. According to WHO data, about 240 million people are chronically infected with hepatitis B. The estimated number of annual death cases due to hepatitis B complications (namely, cirrhosis and liver cancer) exceeds 680,000.

Only mass human vaccination with efficient hepatitis B vaccine created the barrier against the infection, thereby preventing the development of disease complications.

Nevertheless, high prevalence of HBV infection creates a serious problem in certain world regions, e.g. in sub-Saharan Africa, South and East Asia, where 5-10% of the adults is chronically infected

Hepatitis B is ***anthroponotic*** disease. The ***source of infection*** is human carrier of sick person.

The ***infectious dose*** for HBV is ***low*** but not yet firmly established (usually less than 100 viral particles).

The main ***routes*** of HBV transmission are: ***parenteral*** after medical manipulations via blood and its products ("*artificial route*"), ***sexual intercourse***, and ***vertical*** transmission from mother to child.

As the result, HBV infection is a substantial ***occupational hazard*** for healthcare workers due to the elevated risk of their exposure to infected blood and low infectious dose of the pathogen.

Incubation period of hepatitis B is ***long*** and lasts from 1 to 6 months.

When appeared in the bloodstream, HBV enters the liver, binds to membrane *liver bile acid transporter protein* (***LBAT***) by viral HBsAg and replicates within hepatocytes.

It is generally assumed that HBV is not directly cytopathic to infected hepatocytes, but cytotoxic T cells attack the infected hepatic cells. Thus, cellular ***autoimmune reactions*** promote cytolysis of hepatocytes.

Clinical findings of acute HBV infection include fever, nausea and vomiting, jaundice with dark urine and pale stools.

In some cases extrahepatic autoimmune manifestations occur resulting in skin rashes, arthralgias or arthritis. Severe (***fulminant***) forms of hepatitis B develop in 1-2% of patients with lethality above 60%.

The disease has a tendency to ***chronicity*** eventually observed in 5-10% of cases. The risk of chronic infection is highest (up to 90%) for infants infected during pregnancy.

Chronic HBV infection elevates the risk of ***hepatocellular carcinoma***. Therefore, HBV is regarded as established ***biological carcinogen***.

Despite possible autoimmune injury of hepatocytes, T cell-mediated ***immunity*** is considered to be essential for patient recovery. Elimination of infected cells and inhibition of viral replication through the release of cytokines, e.g., γ-interferon and TNF-α, is a cornerstone of viral clearance during acute HBV infection. It is generally assumed that chronic infection is related to a weak T cell response to viral antigens.

Binding of specific antibodies to viral envelope antigens also contributes to clearance of the virus.

Laboratory Diagnosis

Viral DNA, antigens and antiviral antibodies are revealed in blood and liver biopsy ***specimens***. HBsAg can be detected also in saliva, semen, vaginal secretions, etc.

The most valuable tests are ***ELISA*** for detection of HBV antigens and antibodies and ***PCR*** for viral DNA.

HBsAg indicates HBV infection – acute or chronic. HBsAg is usually detectable 2-6 weeks prior to clinical and biochemical signs of hepatitis. It persists throughout the clinical course of the disease but typically disappears by the 6^{th} month after exposure.

HBeAg correlates with active HBV replication and high serum load with hepatitis B viruses

Anti-HBs antibodies indicate past infection with immunity to HBV, or immune response to hepatitis B vaccine.

High levels of IgM ***anti-HBc***-specific antibodies are frequently determined at the onset of clinical illness.

Anti-HBe antibodies are present in serum of persons with chronic HBV infection with low titers of HBV.

Viral DNA detection by PCR correlates with active viral replication.

Principles of Disease Treatment and Prophylaxis

Hepatitis B is treated with reverse transcriptase inhibitors (e.g., ***lamivudine***) and inhibitors of viral DNA synthesis (***telbivudine***).

Recombinant interferon-alpha shows beneficial effect in the treatment of patients with chronic HBV infection.

For ***passive post-exposure prophylaxis*** hepatitis B ***immune globulin*** is administered within 12 hours after percutaneous exposure to HBsAg-positive blood, or after sexual intercourse with HBsAg-positive person.

For ***active specific prophylaxis*** HBsAg ***recombinant vaccine*** is effectively used worldwide. HBV vaccination is an essential part of national immunization programs in many countries.

Primary injection of vaccine is made during 12 h after birth; two boosters are conducted in 1 and 6 months. Full vaccination course confers specific immunity to 99% of persons.

Pre-exposure prophylaxis with hepatitis B vaccine is conducted for all susceptible high-risk groups, including medical personnel. The protective immunity lasts for 10-15 years.

Non-specific prophylaxis includes the procedures that can limit the risk of infection to healthcare workers, laboratory personnel, and other susceptible individuals. All blood remnants, body fluids and contaminated materials should be treated as if they are infectious for HBV, HCV, HIV, and other parenteral viruses. The medical instruments are sterilized by autoclaving.

HEPATITIS DELTA VIRUS

The History of Virus Discovery

From time to time highly severe (***fulminant***) hepatitis develops in patients with HBV infection. It has been demonstrated that fulminant HBV disease is associated predominantly with patient superinfection by new hepatitis D or ***hepatitis delta virus*** (***HDV***).

HDV was first isolated from patients with severe hepatitis B by M. Rizetto in 1977.

Classification and Structure of HDV

HDV is not included into any viral family (unclassified virus). It is placed into separate genus *Deltavirus* with single species ***hepatitis delta virus***.

Hepatitis D virion is ***spherical*** particle with ***icosahedral symmetry***, about 40 nm in size. Genome of HDV consists of very small ***circular negative single-stranded RNA***, 1.7 kb in size. Genomic RNA encodes intrinsic HDV capsid antigen (***HDAg*** or ***delta Ag***).

No homology exists between HBV and HDV genomes.

HDV is a ***defected satellite virus*** (subviral agent) that reproduces only in presence of ***helper*** hepatitis B virus.

HDV requires HBsAg of hepatitis B virus – together with host cell lipids HBsAg creates ***envelope*** for HDV virion.

Viral Replication Cycle

HDV exploits the same membrane receptor as HBV (*liver bile acid transporter protein* or ***LBAT***) to infect hepatocytes. After uncoating HDV nucleocapsid is transported into the cell nucleus, where genome replication occurs.

HDV uses cellular RNA polymerases to create circular genomic RNA and linear mRNA for translation of protein HDAg.

HDAg binds to viral genomic RNA transported into cytoplasm resulting in nucleocapsid formation. It acquires external coat from proteins of HBsAg of hepatitis B virus and releases via cytoplasmic membrane of hepatocytes.

Thus, productive HDV infection is possible only in case of hepatocyte co-infection with HDV and HBV agents.

Characteristics of HDV Infection

As estimated, about 15-20 mln people are currently coinfected with HDV and HBV worldwide. HDV infection is often associated with the most severe forms of hepatitis in HBsAg-positive patients. Chronically infected individuals demonstrate high risk of progression to end-stage liver disease, resulting in cirrhosis and hepatic carcinoma.

Intravenous drug abusers and persons, who received multiple hemotransfusions, are of high risk of HDV infection.

The primary ***routes of transmission*** for HDV are similar to HBV, though HDV is not a sexually transmitted disease. Perinatal transmission of HDV is rare.

The ***incubation period*** varies from 2 to 12 weeks.

While HDV is absolutely dependent on a coexistent HBV infection, acute hepatitis D may develop either as a simultaneous infection (***coinfection***) with HBV or as a ***superinfection*** of patient with primary chronic HBV infection.

Coinfection is similar to acute hepatitis B resulting in viral elimination in more than 90% of cases with complete recovery.

By contrast, superinfection by HDV is frequently associated with severe acute (“fulminant”) hepatitis that, if recovered, results in chronic HDV disease (near 90% of cases). Fulminant hepatitis D is a life-threatening disorder with fatality of 70-90%.

Laboratory Diagnosis

Serological tests are used for hepatitis D diagnosis. Both HDAg and anti-delta IgM antibodies are determined by ***ELISA*** in case of acute delta infection.

Also HDV RNA is discovered in patient's serum by ***PCR***.

All markers of HDV replication decline to convalescence, but HDV antibodies may disappear within months or years.

Treatment and Prophylaxis of hepatitis D

Recombinant interferon-alpha is commonly used for ***treatment*** of delta hepatitis. Antiviral chemotherapy of HDV infection is currently not elaborated.

In healthy individuals delta hepatitis can be fully prevented by ***vaccination*** of persons with hepatitis B vaccine. However, vaccination doesn't protect chronic hepatitis B carriers from superinfection by HDV.

HEPATITIS C VIRUS

The History of Virus Discovery

Long time accumulated clinical, epidemiological and laboratory data strongly indicated that it should be widespread hepatitis agent not related to HAV or HBV. Intensive attempts to identify enigmatic pathogen were protractedly unsuccessful until M. Houghton with colleagues, D. Bradley and H. Alter in 1985-1989 by the methods of molecular cloning isolated and then sequenced nucleic acid of a novel causitive agent of viral hepatitis, later designated as hepatitis C virus or HCV.

Classification

Hepatitis C virus (***HCV***) belongs to *Flaviviridae* family. Currently this family contains 4 viral genera. The genus with the most numerous viral species here is *Flavivirus*. It harbors at least 53 zoonotic viral species that pertain to *arboviruses*, contracted by arthropod vectors to animal and human hosts.

Genus *Hepacivirus* contains single species of ***hepatitis C virus***.

HCV demonstrates ***extreme genetic variability*** being divided into 7 ***genotypes***, each contains multiple genetic ***subtypes*** (totally more than 100). Subtype variations generate HCV ***quasispecies*** during the course of individual infection.

HCV subtypes ***1a*** and ***1b*** have global distribution, they account for about 60% of total infection cases.

Structure of HCV

HCV is a ***positive single-stranded RNA*** virus of ***spherical*** shapes with lipid ***envelope***, 60 nm in size. HCV virion has ***icosahedral symmetry***.

Viral particle has a ***core*** protein of nucleocapsid, two envelope glycoproteins ***E1*** and ***E2***.

Proteins ***E1*** and ***E2*** promote HCV binding and fusion with membranes of hepatocytes facilitating viral entry.

Viral genomic RNA encodes a number of non-structural proteins (***NSP2- NSP5A*** and ***NSP5B***) with multiple functions.

They possess enzymatic activity (*helicase*, *protease*), inhibit host interferon production, suppress lymphocyte activation, or induce apoptosis of infected cells.

Non-structural protein ***NSP5B*** is viral ***RNA polymerase***.

Hepatitis C viruses can't propagate in laboratory cell lines except hepatocyte cultures.

Virion Resistance

HCV as all flaviviruses is relatively unstable in the environment. Nevertheless, in plasma, if exposed to room temperature or dried, the virus maintains viability at least for 16 h. Viral survival expands at low temperatures.

Heating at 60-70^{o}C for 10 minutes irreversibly inactivates virus.

HCV is sensitive to UV light and all conventional disinfectants (halides, aldehydes, phenol, H_2O_2, detergents, ether, ethanol, etc.)

Viral Replication Cycle

HCV infects hepatocytes, but it may replicate within lymphocytes and monocytes.

Virus attachment is performed via binding of E1 and E2 viral proteins to broad group of cell membrane receptors. Viruses enter hepatocytes by ***endocytosis***. Viral uncoating is facilitated with endosome acidification resulting in fusion of lipid envelope with endosomal membrane.

All cycle of virus propagation occurs in ***cytoplasm*** of infected cells.

Genomic (+) RNA translation results in primary *polyprotein* synthesis that further processed by viral and cellular proteases into mature viral proteins.

Viral RNA polymerase ***NSP5B*** catalyzes genomic RNA replication through the stage of negative-sense RNA intermediate, which serves as template for genomic (+) RNA synthesis.

Virion assembly and maturation is finalized by release of progeny viruses that leave hepatocytes by ***budding***.

Pathogenesis, Clinical Findings and Immunity in Hepatitis C

Current infection by HCV demonstrates high prevalence throughout the world. According to WHO data, about 3% of human population are infected with HCV. Furthermore, some population subgroups (e.g., in Africa) have prevalence rates as high as 10%. Other high-prevalence areas are found in South America and Asia.

It is estimated that totally there are more than 170 million HCV chronic carriers, who have elevated risk of progression of end-stage liver disease with cirrhosis, or hepatic carcinoma.

Hepatitis C is solely ***anthroponotic*** disease. The ***source of infection*** is human (chronic patient or carrier).

Incubation period usually lasts from 1 to 3-4 months.

The main ***route of transmission*** is ***parenteral***. The major risk groups are ***intravenous drug abusers*** that apply non-sterile syringes for drug injections, and persons, undergoing frequent massive blood transfusions. Sexual or vertical transmission is much more seldom (less than 5%).

Most new infections with HCV are subclinical. Hospitalization is rare, and jaundice occurs in less than 25% of patients.

Self-recovery after primary HCV infection greatly varies in the range near 20-40%.

The majority (more than 60%) of HCV patients develops ***primary chronic hepatitis***, and many are at the risk of progressing towards chronic active hepatitis and cirrhosis (10-20%). About 40% of chronic liver disease is HCV-related.

Also HCV infection substantially elevates the risk hepatocellular carcinoma' i.e., HCV is a potent biological ***carcinogen***.

HCV is regarded to exert low or moderate cytopathic effect (e.g., liver steatosis) but hepatocytes are damaged by cellular ***autoimmune reactions***. Many isolates of HCV are resistant to interferons, as viral proteins can block interferon-inducible antiviral cell proteins.

In number of chronic HCV infections ***extrahepatic manifestations*** arise resulting in skin rashes, arthritis, progression of diabetes mellitus, etc.

During the long course of chronic infection HCV undergoes active mutational process. It is essential for fast propagating RNA-containing viruses with *error-prone replication* with high mutation rate.

This results in generation of a great number of mutant genetic variants (***quasispecies*** or "***mutant cloud***") arisen from primary HCV virus in the course of individual viral infection.

The emergence of quasispecies is extremely significant in progression of viral disease. The ongoing pressure from the side of host immune system and the influence of antiviral drugs promote the selection of resistant viral mutants. This leads to creation of new genetic lines resistant to antiviral therapy and therefore, leads to the progression of viral disease.

Overall, in many patients the ***immunity*** almost fails to prevent chronic infections by HCV. What's more, the virus actively develops genetic variations during chronic infections resulting in change of viral antigenic structure.

Laboratory Diagnosis of Hepatitis C

Serological testing is commonly elaborated for diagnosis of HCV infection. ***ELISA*** determines ***antibodies to HCV*** but don't distinguish between acute, chronic, or resolved infection.

Nucleic acid-based assays (mainly, reverse transcriptase ***PCR***) detect the presence of circulating HCV RNA. Quantitative real-time ***PCR*** is essential monitoring patients on antiviral therapy.

Nucleic acid hybridization is used to determine genotypes of HCV isolates as well as for detection of viral RNA in liver biopsy specimens.

Principles of Disease Treatment and Prophylaxis

Active specific prophylaxis of HCV infection by vaccination is ***not available*** now. Great difficulties in design of HCV vaccines are inevitable due to high genetic diversity of hepatitis C virus.

Prior to 2013, combination therapy of hepatitis C patients with recombinant *interferon-alpha* and antiviral drug *ribavirin* was regarded as the method of choice for treatment of HCV infection. But only about 40-80% of chronic HCV patients depending on HCV genotype responded to this therapy regimen that lasted up to 48 weeks. The treatment was also followed by serious side effects. The results were improved with administration of prolonged interferons conjugated with polyethyleneglycol (*PEG-interferons*).

Fortunately, almost simultaneously in 2013/2014 novel highly efficient anti-HCV drugs were introduced into clinical practice that literally revolutionized the treatment of this life-threatening infection.

With highest specificity they arrest the activity of viral proteins essential for HCV replication. In particular, ***simeprevir*** blocks viral ***protease*** action, ***sofosbuvir*** inhibits ***viral RNA polymerase NS5B***, and ***ledipasvir*** inactivates non-structural protein ***NS5A***, responsible for viral replication, assembly and egress.

Taken in various combinations, they increased treatment success rate above 95% even for the most resistant 1 genotype of hepatitis C virus.

The only serious obstacle still impeding the broad use of these life-saving drugs is a very high cost of treatment course.

Chapter 18
RETROVIRUSES: HUMAN IMMUNODEFICIENCY VIRUS (HIV)

HUMAN IMMUNODEFICIENCY VIRUS

The History of HIV Discovery

Human immunodeficiency virus (***HIV***), supposed to be derived from primate lentiviruses, is the etiological agent of ***HIV infection*** with ***acquired immunodeficiency syndrome***, or ***AIDS***.

The infection was first registered in 1981, and its causative retroviral agent was primarily isolated by the end of 1983 by French virologists F. Barrй-Sinoussi and L. Montagnier. To confirm the results of discovery, this viral isolate was sent to highly experienced US laboratory headed by R. Hallo that specialized in retrovirus study. At the end of 1983 the research group of F. Wong-Staal working under the guidance of R. Hallo reported about isolation of a new retrovirus – putative causative agent of AIDS. Further molecular genetic analysis demonstrated the identity of genomes of both viral agents.

In 2008 F. Barrй-Sinoussi and L. Montagnier were awarded Nobel Prize in Physiology or Medicine for their pivotal discovery.

In 1986 in West Africa F. Clavel and coworkers discovered another species of human immunodeficiency virus – HIV-2.

Classification of Retroviruses

Retroviridae family of RNA-containing viruses embraces 2 subfamilies (*Orthoretrovirinae* and *Spumaretrovirinae*) with 7 viral genera.

All the representatives of family *Retroviridae* contain unique ***reverse transcriptase*** enzyme, which synthesizes DNA copy of viral genome on the base of viral RNA template.

HIV viruses (species ***HIV-1*** and ***HIV-2***) belong to subfamily *Orthoretrovirinae* and *Lentivirus* genus, which encompasses the viruses, capable of causing long-lasting *slow viral infections*.

Lentiviruses have been isolated from many living species, including 20 different primate species.

As some other RNA-containing viruses, HIV demonstrates outstanding ***genetic variability***. HIV-1 is divided into genetic ***groups*** (M, N, O, and P). More than 90% of cases of HIV-infection are caused by representatives of ***M*** (***main***) viral group.

Every genetic group contains multiple genetic ***subtypes***. For instance, M group includes above 10 subtypes (A-K). Subtypes are able to cross-recombination with formation of *circulating recombinant forms* of HIV.

HIV-2 has 9 genetic groups (A-I).

Subtype variations generate multiple HIV ***quasispecies*** during the course of HIV infection.

Structure of HIV

HIV viruses are ***spherical*** in shape 80-100 nm in diameter with cylindrical cone-shaped core. Virion is covered with lipid ***envelope***, carrying receptor glycoproteins. Under lipid coat protein shell of p17 protein is present.

Viral genome is composed of ***single stranded positive-sense RNA***. The genome is diploid that means the presence of two equal (+) RNA molecules.

HIV contains three genes encoding viral structural proteins – ***gag***, ***pol***, and ***env***.

Viral ***env gene*** encodes major viral envelope proteins. Glycoprotein ***gpl20***, product of *env* gene, contains binding domains for virus attachment to host ***CD4 molecules*** and ***coreceptors***, and carries the main antigenic determinants that elicit neutralizing antibodies.

Glycoprotein ***gp4l*** of *env* gene contains both a transmembrane domain that anchors the glycoprotein gp120 in the viral envelope and a fusion domain that facilitates virus penetration into target cells. Combination of gp41 and gp120 results in complex ***gp160*** glycoprotein, integrated in the viral envelope.

Viral ***pol gene*** codes for viral enzymes ***reverse transcriptase*** (*p66*), ***protease*** (*p32*), and ***integrase*** (*p11*).

Gene gag (group specific antigen) encodes primary polyprotein with inner capsid peptides (e.g., ***p17*** and ***p24***). After translation viral polyprotein is cleaved by viral protease resulting in structural proteins.

Six additional genes encode proteins that regulate viral reproduction *in vivo* participating in pathogenesis of HIV infection:

– *Tat* protein (product of *tat* gene) activates reverse transcription of viral RNA;

– *Rev* protein (product of *rev* gene) regulates transcription of viral structural proteins;

– *Nef* protein (product of *nef* gene) inhibits expression of CD and HLA molecules on membranes of infected cells; elicits chemokine production by macrophages that activate resting T cells resulting in productive HIV infections;

– *Vif* protein (product of *vif* gene) suppresses synthesis of antiviral proteins;

– *Vpr* protein (product of *vpr* gene) stimulates replication of viral RNA and transcription of viral protens;

– *Vpu* protein (product of *vpu* gene) promotes virion release;

– *Vpx* protein of HIV-2 (product of *vpx* gene) stimulates reverse transcription of HIV-2 RNA.

Virion Resistance

Despite generally low environmental resistance of retroviruses, in certain conditions and in high concentrations HIV maintains viability for a long time (e.g., above 10 days in blood in syringes at room temperature or at 4^{o}C for 2-4 weeks). When dried in blood spots, HIV stays viable for several days.

HIV remains infectious during long-term storage at low temperatures (-20^{o}C and less). Virus is inactivated by heating at 60°C for 30 min and at 100^{o}C for 1 min.

HIV is sensitive to the most common disinfectants and antiseptics (household bleach, sodium hypochlorite and other halides, hydrogen peroxide, aldehydes, ethanol, detergents, and others). The virus is also killed by acidic or alkaline pH.

Viral Replication Cycle

Virus attachment is performed via ***gp120 receptor*** that binds to ***CD4 molecules*** of lymphocytes, macrophages, and some other cell types.

A highest density of membrane CD4 is essential for $CD4^+$ ***T helper cells*** and ***T memory cells***, but expression of CD4 on monocytes, ***macrophages***, dendritic cells, ***glial cells***, astrocytes and neurons of CNS, or enterocytes is enough for HIV adherence.

A ***second co-receptor*** in addition to CD4 is necessary for HIV-1 to gain entry to the cells. It is required for fusion of the virus with the cell membrane. Virus first binds to CD4 and then to the coreceptor. These interactions cause conformational changes in the viral envelope that induces gp41-mediated membrane ***fusion*** and virus uptake.

Host ***chemokine receptors*** serve as HIV co-receptors – ***CCR5*** is the predominant co-receptor for macrophage-tropic strains of HIV, whereas ***CXCR4*** is the co-receptor for lymphocyte-tropic strains of HIV.

Uncoating of HIV occurs in cytoplasm. Here viral genomic RNA undergoes ***reverse transcription*** to complementary DNA by enzyme ***reverse transcriptase***.

Viral DNA is imported to the nucleus for integration to the host genome (***provirus state***). Incorporation of HIV DNA is catalyzed by viral ***integrase***.

HIV replication largely occurs in ***activated T cells*** and to a lesser extent in macrophages. They are stimulated by ***TNF-alpha*** and other proinflammatory cytokines followed by activation of cellular transcription factors (mainly, ***NF-κB***) that trigger the transcription of viral RNA.

After ribosomal translation primary viral polyproteins are processed by viral ***protease***.

Viral assembly (morphogenesis) takes place in the cytoplasm; progeny virions leave the cell by ***budding***.

Complete duration of HIV replication cycle averages 2.5 days.

As it was mentioned above, HIV demonstrates ***highest genetic variability***. Because of rapid viral proliferation and the error-prone mode of activity of HIV reverse transcriptase, every nucleotide of the HIV genome probably undergoes daily mutation.

Epidemiology, Pathogenesis, Clinical Findings and Immunity in HIV Infection

From its first case, HIV infection and AIDS has become a global epidemic, affecting different populations and all geographic regions. Once infected, individuals remain infected lifelong. Within a decade, if left

untreated, the vast majority of HIV-infected persons develop fatal opportunistic infections resulting from HIV-induced suppression of the immune system.

The ***source of infection*** is *HIV-infected person*.

The most common ***route of transmission*** is ***sexual intercourse*** (*homo-* and *heterosexual* contacts) that accounts for more than 70% of infection spread.

Next follows ***parenteral transmission*** (*artificial route*) that predominantly includes ***intravenous drug use***. Occasionally the infection is contracted after ***medical manipulations***, predominantly, blood transfusions.

Mother-to-child or ***vertical transmission*** occurs during pregnancy or childbirth, and through breastfeeding with maternal milk.

Transmission of HIV-2 is 10-20 times less frequent than HIV-1 in the same conditions of exposure to virus.

HIV infection affects ***CD4-bearing*** cell populations.

The main target of virus action is the subset of ***T helper*** lymphocytes, which express ***CD4*** phenotypic marker on their surface.

It was determined that HIV co-receptor ***CXCR4*** is carried by "naive" T cells, whereas another co-receptor ***CCR5*** is expressed predominantly by macrophages and dendritic cells as well as by activated T lymphocytes and memory T cells.

After viral entry ***reverse transcription*** of viral genomic RNA leads to complementary DNA synthesis and its further integration with cell genome resulting in ***provirus state***.

Proviruses remain long-time associated with affected cells, but under the number of external and internal stimuli viral replication is activated. Various cytokines (e.g., ***TNF-alpha*** or IL-10) induce HIV transcription in both macrophages and T cells.

Chronic activation of the TNF-signaling pathway enhances HIV-1 transcription. Viral propagation within T helpers causes cell death due to multiple devastating effects of virus:

– cytopathic effect on T cells with *syncytium* formation;

– direct and TNF-mediated *apoptosis* of T cells;

– *pyroptosis*, or inflammatory T cell death via activation of caspase-1;

– *death* of infected T cells under the attack of cytotoxic T cells and NK cells;

– general inhibition of *hematopoiesis.*

Monocytes and macrophages play a substantial role in the dissemination of HIV infection. Unlike $CD4^+$ T lymphocytes, monocytes are relatively refractory to the cytopathic effects of HIV, and the virus can be transported to various organs and tissues (such as the lungs and brain).

Infection of glial cells, astrocytes and neurons of CNS is followed by permanent neuropsychiatric disorders in patients with HIV infection.

Progressive decline of T helpers leads to profound attrition of the immune system that affects basic functions of cytotoxic T cells, natural killer cells, impairs secretion of cytokines, etc.

As the final result, a tremendous variety of ***opportunistic infections*** arises in HIV-infected person due to deep immunosuppression, caused by HIV.

Typical course of untreated HIV infection spans about a decade. Stages of HIV infection include:

– ***incubation period***;
– ***acute infection***;
– ***clinical latency***;
– ***persistent generalized lymphadenopathy***;
– ***AIDS*** development;
– ***death*** of patient resulted from AIDS-associated diseases.

During ***incubation*** in 24 h after exposure HIV invades dendritic cells at the portal of entry; in 24-48 h infected dendritic cells migrate into regional lymph nodes; in 4-11 days HIV appears in blood resulting in ***viremia***; and in 3-4 weeks first clinical signs of infection arise.

Acute infection is characterized with ***dissemination of virus*** to lymphoid organs. An ***acute mononucleosis-like syndrome*** develops in many patients (50-75%) 3-6 weeks after primary infection. There is a significant drop of circulating CD4 T cells at early time. An immune response to HIV occurs 1 week to 3 months after infection, plasma viremia declines, and the number of CD4 cells restores. However, the immune response is unable to clear the infection completely, and HIV-infected cells persist within the lymph nodes.

The period of ***clinical latency*** may last for as long as 10 years. During this time, there is a high level of ongoing viral replication.

Coming next ***persistent generalized lymphadenopathy*** is characterized with gradual enlargement of different groups of lymph nodes.

And eventually, the patient will develop clinically manifested disease with ***opportunistic infections*** or ***neoplasms***. They emerge due to severe

immune system failure resulting in ***acquired immunodeficiency syndrome***, or ***AIDS***.

The predominant sources of morbidity and lethality among AIDS patients are ***opportunistic infections***, induced by pathogenic agents that rarely cause serious diseases in immune-competent individuals.

The most common opportunistic infections in AIDS patients are caused by:

bacterial pathogens – ***Mycobacterium tuberculosis***, *Mycobacterium avium-intracellulare*, *Listeria monocytogenes*, salmonella species, streptococcus species and many others;

viruses – cytomegalovirus (***CMV***), varicella-zoster virus, herpes simplex viruses, adenoviruses, etc.;

protozoans – *Toxoplasma gondii, Cryptosporidium spp.*;

fungi – *Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum*, and *Pneumocystis jirovecii*.

Tuberculosis is the major AIDS-associated disease in HIV-infected persons. It develops at least in 30-40% of HIV-infected individuals being the major cause of patient's death (25-40% of total AIDS lethality). Global spread of HIV and tuberculosis co-infection is sometimes called as "***Syndemic***".

Severe ***AIDS-indicator diseases*** are also associated with mycobacterioses, systemic candidiasis, CMV- and varicella-zoster viral infections, pneumocystis pneumonia and *T. gondii* infection.

AIDS patients exhibit a marked predisposition to the development of cancer. AIDS-associated tumors include ***lymphomas***, ***Kaposi's sarcoma***, (vascular neoplasm, caused by herpesvirus type 8, thought to be of endothelial origin), cervical cancer, etc.

Neurological abnormalities are common in AIDS affecting 40-90% of patients. They are manifested by HIV ***encephalopathy***, ***peripheral neuropathies***, and most serious, ***AIDS dementia complex***.

HIV-infected persons develop both humoral and cell-mediated ***immunity*** against the virus. Antibodies to a number of viral antigens (p24, gp41, gpl20) develop soon after infection, but they can't stop infection progressing. Cellular responses (cytotoxic T cells and natural killers) can destroy virus-infected cells.

Nevertheless, immune response to HIV ***isn't able to eliminate virus***.

AIDS consequences, predominantly opportunistic infections and CNS disorders, lead to inevitable ***death of AIDS patient***.

Evident devastating nature of HIV infection multiplied by its rapid spread has posed a tremendous threat to global health security.

In 2005 Joint United Nations Programme on HIV/AIDS (***UNAIDS***) in its annual special report characterized HIV infection as "...one of the most destructive epidemics in recorded history".

On the peak of AIDS pandemic (1996-2005) a total number of HIV-infected persons became closer to 40 million, about 2-3 million people annually died from AIDS-associated diseases, and the annual number of individuals newly infected with HIV exceeded 4 million.

According to WHO data, more than 34 million people died from the start of AIDS pandemic.

In September 2000 Member States of United Nations at the Millennium Summit adopted United Nations Millennium Declaration. It included 8 crucial international development aims – the Millennium Development Goals (MDG).

The Goal 6 claimed "...***to halt and begin to reverse the spread of HIV/AIDS by 2015***". It was appended in 2006 with a new AIDS target "...to achieve universal access to antiretroviral medicines for people in need by 2010".

As the result, only owing to outstanding joint efforts of national governments and global international organizations working under the aegis of UNAIDS (WHO, UNESCO, UNDP, UNICEF, World Bank and others) it has become possible to break down the progression of HIV pandemic.

This great success was largely related with expanding availability of highly efficient antiretroviral therapy for HIV-infected people.

In July 2015 the report of UNAIDS entitled "How AIDS changed everything" with preface of UN General Secretary Ban Ki-moon stated: "The world has achieved the AIDS targets of Millennium Development Goal 6. ***The epidemic has been halted and reversed***".

It has been confirmed by the next data – the number of persons newly infected with HIV globally declined by 35% in 2000–2014; the number of people dying from HIV-related diseases declined by 41% since 2004, the peak epidemic year; and almost 16 million people acquired HIV treatment.

Current number of HIV-infected individuals is equal now to 36.9 million.

UNAIDS report has also emphasized a future challenging objective as essential part of the sustainable development goal – ***ending the AIDS epidemic by 2030***.

Despite undoubt achievements in the combat with HIV infection, a lot of issues haven't been solved yet.

First of all, a total number of HIV-infected persons still remains great. The most of suffering people (estimated 25.8 million) live in Sub-Saharan Africa, the region "most heavily affected by the epidemic". An estimated 5 million HIV-infected people live in Asia and the Pacific region.

Second, antiretroviral treatment should cover at least 60-80% of all HIV-infected persons to maintain positive tendency of global HIV decline.

And finally, certain world regions demonstrate increasing trends in HIV spread; among them are the countries of Eastern Europe and Central Asia, including Russian Federation, Ukraine, and Belarus.

Laboratory Diagnosis of HIV Infection

Diagnosis of HIV infection is established only on the base of highly specific and sensitive laboratory tests that detect specific antiviral antibodies or viral nucleic acid in ***clinical specimens*** (primarily, patient's blood or serum).

Serological testing is the most suitable in clinical practice to determine HIV infection. Antibodies to HIV are evaluated by enzyme-linked immunosorbent assay (***ELISA***). If properly performed, these tests have a sensitivity and specificity exceeding 98%.

The first positive ELISA test of a serum sample must be confirmed by a ***repeat testing***.

If the repeat ELISA is reactive, a ***confirmatory test*** is performed. The most wide used confirmation assay is the ***western blot*** technique, in which antibodies to various HIV proteins of specific molecular weights should be simultaneously detected. Antibodies to viral core protein p24 or envelope glycoproteins gp41, gpl20, or gpl60 are most commonly assessed.

Simple and rapid tests for detecting HIV antibodies are based on ***latex-agglutination***.

The majority of individuals display ***seroconversion*** (reveal antibodies) within 2 months after viral exposure. HIV infection for longer than 6 months without a detectable antibody response is very rare.

Amplification assays such as the ***real-time reverse transcriptase PCR*** and hybridization tests are also commonly used to detect viral RNA in clinical specimens. HIV RNA level (***viral load***) is an important predictive marker of disease progression and valuable tool to monitor the efficacy of antiviral therapy.

Other auxiliary laboratory tests for AIDS diagnosis include various methods of assessment of immune status. ***CD4$^+$ T helper subset count*** is evaluated by flow cytometry or immunofluorescence with monoclonal anti-CD4 antibodies.

CD4$^+$ cell count (normal level – 800-1000 cells/μL of blood) shows direct correlation with AIDS progression – falling below 500 cells/μL predisposes to development of opportunistic infections; the decline less than 200 cells/μL corresponds to AIDS.

Diagnosis of ***AIDS-associated opportunistic infections*** rests on rapid ***molecular tests*** (e.g., PCR) followed by pathogen isolation and identification.

Principles of HIV Treatment and Prophylaxis

Practical implementation of HIV chemotherapy with combinations of various antiretroviral drugs, referred to as ***highly active antiretroviral therapy*** (***HAART***), literally revolutionized the treatment of HIV-infected persons.

HAART suppresses viral replication below the limits of detection in plasma and decreases the viral load in lymphoid tissues that leads to the recovery of immune response.

However, HAART has failed to cure HIV infection. DNA copy of viral genome is harbored within long-living infected cells, including memory CD4$^+$ T cells and probably macrophages and seminal cells. When HAART is discontinued or there is a treatment failure, virus production reactivates. Thus, the treatment should last lifelong.

Nonetheless, if HIV patients strictly follow treatment regimens, their life expectancy comes closer to average lifespan of human population.

Antiretroviral drugs with various mechanisms of action are included into HAART treatment scheme:

– ***nucleoside inhibitors*** of HIV ***reverse transcriptase*** (***lamivudine***, abacavir, azidothymidine and many others);

– ***non-nucleoside inhibitors*** of HIV ***reverse transcriptase*** (***efavirenz***, nevirapine);

– HIV ***protease inhibitors*** (***indinavir***, ***nelfinavir***, ritonavir);

– HIV ***integrase inhibitors*** (***raltegravir***, elvitegravir);

– ***inhibitors*** of HIV ***entry*** and ***fusion***:

– ***maraviroc*** – inhibits binding of gp120 to human CCR5 co-receptor;

– ***enfuvirtide*** – binds to HIV gp41 receptor, thereby blocking viral fusion.

Initial HAART schedule presumes administration of 2 ***nucleoside inhibitors*** of HIV ***reverse transcriptase*** and one drug from other antiretroviral groups (e.g., HIV protease inhibitor).

Whereas monotherapy usually results in the rapid emergence of drug-resistant mutants of HIV, combination therapy, which targets multiple steps in virus replication, prevents the emergence of HIV quasispecies.

Post-exposure prophylaxis of HIV infection (***PEP***) is administered after occasional contact with biological fluids of HIV-infected persons (unprotected sexual intercourse, accidental needlestick injury, scalpel cut, infected blood transfusion, etc.) To be maximum effective, PEP must be initiated without any delay (within first 72 hours after the exposure). Modern schemes based on HAART are currently used including HIV integrase inhibitors. After primary PEP it is recommended to continue antiretroviral therapy at least for 4 weeks. The repeat laboratory testing of affected persons is performed up to 6 months after exposure.

Specific prophylaxis of HIV infection is not created yet. A safe and effective vaccine is the best tool of controlling the AIDS pandemic. Recombinant viral envelope glycoproteins are the most likely candidates for vaccine.

Unfortunately, vaccine development is extremely difficult because HIV mutates rapidly; furthermore, the virus stays integrated within genome of many infected cells and thereby not completely eliminated by host immune response.

Non-specific prophylaxis presumes the maintainance of a lifestyle that minimizes or eliminates the high-risk factors of HIV spread. This is directly related with the success of educational projects offering behavioral changes.

Chapter 19
HERPESVIRUSES AND HERPES VIRAL INFECTIONS

The History of Discovery of Herpes Viruses

Herpetic infections (Gr. *herpes* means "to creep or crawl") are the commonest among humans. They follow humankind throughout all its history. It is generally ascertained that more than 90% of human population are infected with herpesviruses.

The main representatives of herpesiviruses were identified in XX century. At first, A. Lowenstein discovered herpes simplex virus in 1919. Then in 1925 K. Kundratitz confirmed the link between varicella and zoster infections; H. Ruska in early 1940s found this virus by electron microscopy, and finally the varicella-zoster agent was isolated in 1952 by T. Weller.

In 1955-1956 M. Smith obtained the culture of human cytomegalovirus (CMV).

In 1964 M. Epstein, B. Achong, and Y. Barr established a new herpes virus isolated from Burkitt's lymphoma cells that was later termed as Epstein-Barr virus (EBV). Further in 1968 W. Henle and G. Henle identified Epstein-Barr virus as the causative agent of infectious mononucleosis.

First lymphotropic human herpesvirus type 6 was primarily isolated in 1986 by S.Z. Salahuddin and coworkers in laboratory of R. Gallo; another herpesviral species of type 6 was revealed by the group of K. Yamanishi in 1988.

Next lymphotropic human herpesvirus of type 7 was described by N. Frenkel and coworkers in 1990.

The most recently found Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 was first detected in 1994 by Y. Chang, P. Moore, and colleagues.

Classification of Herpesviruses

Herpesviruses are placed into order *Herpesvirales* and *Herpesviridae* family; the latter comprises multiple viral genera and species of human and animal herpesviruses. To date, ***8 types*** of human herpesviruses are known.

Family ***Herpesviridae*** is divided into 3 subfamilies.

Subfamily *Alphaherpesvirinae* includes genus ***Simplexvirus*** with species human alphaherpesvirus 1 (***herpes symplex virus type 1***) and human alphaherpesvirus 2 (***herpes symplex virus type 2***) as well as genus ***Varicellovirus*** with species human alphaherpesvirus 3 or ***varicella-zoster virus*** of ***type 3***.

Subfamily *Betaherpesvirinae* comprises genera ***Cytomegalovirus*** (species human betaherpesvirus 5, or ***type 5***, or ***CMV***) and ***Roseolovirus*** (species human betaherpesvirus ***6A*** and ***6B*** of ***type 6***).

Finally, one more subfamily *Gammaherpesvirinae* contains genus ***Lymphocryptovirus*** with species human gammaherpesvirus 4 known as ***type 4*** or ***Epstein-Barr virus*** (***EBV***) and genus *Rhadinovirus* with species human gammaherpesvirus 8 also known as ***herpesvirus type 8*** or ***Kaposi's sarcoma-associated herpesvirus*** (***KSHV***).

Structure of Herpesviruses

Herpesviruses are ***double-stranded linear DNA***-containing ***enveloped viruses***.

Herpesvirus size is ***large*** (about 150-200 nm). The mature virion is of ***spherical*** shape.

Viral nucleocapsid exhibits ***icosahedral symmetry***. It is composed of 162 capsomers. Viral envelope is derived from nuclear membrane of the infected cell and carries viral glycoprotein spikes.

The protein coat between the capsid and envelope is termed as ***tegument***.

There is generally low DNA relatedness between various species of herpesviruses except for herpes simplex types 1 and 2 with 50% sequence homology, and herpesviruses of 6 and 7 types, which show limited (30-50%) genomic DNA similarity. DNA homology predisposes to antigenic cross-reactivity between related herpesviruses.

Genomic DNA of herpesviruses is large. Typical herpesviral genome encodes above 100 viral proteins.

More than 35 polypeptides are included into the structure of mature virion. Mutiple glycoproteins of spikes, protruding outside the envelope (e.g., gB and gD proteins of herpes simplex virus) promote virion binding to cell receptors. Some glycoproteins (e.g., gG protein) confer virus antigenic specificity

A number of ***virus-specific enzymes*** (e.g., DNA polymerase and thymidine kinase) are synthesized in infected cells, but they are not incorporated into the virus particles.

Viral Replication Cycle

Primary virus ***binding*** to target cells largely occurs via interaction of envelope glycoproteins with membrane glycosaminoglycans, e.g., ***heparan sulfate***. Viral ***entry*** is promoted by next binding of spike glycoproteins (like ***gD***) to a number of special high-affinity membrane receptors (herpesvirus ***entry mediators***).

The virus enters the cell by ***fusion*** with the cell membrane. The capsid is transported to a nuclear pore. Then ***uncoating*** occurs, and DNA becomes associated with the nucleus.

Viral DNA is transcribed by cellular RNA polymerase II.

At first ***immediate-early*** and ***early viral proteins*** are translated that serve for viral replication and genome expression.

A large number of ***enzymes*** involved in DNA synthesis is produced. They are subjected to inhibition by specific antiviral drugs.

Viral ***host shutoff protein*** arrests cellular protein synthesis degrading host mRNAs.

Next ***late proteins*** are translated. Most of them are ***structural proteins*** of herpesviruses.

Encasing of newly synthesized viral DNAs into empty nucleocapsids occurs in the cell nucleus.

Maturation accomplishes by ***budding*** of nucleocapsid complexes through nuclear membrane. Enveloped progeny virions are then released by exocytosis from the infected cells.

The duration of replication cycle of herpesviruses varies greatly – from 8-16 h for herpes simplex viruses to over 70 h for cytomegalovirus.

Productive infection is followed by cell death with ***lysis***. On the other hand, herpesviruses stay dormant inside infected cells resulting in long-lasting ***latent infection***. In herpes symplex viruses this state is maintained by synthesis of viral latency associated transcript RNA (***LAT***). It influences normal cell life cycle and slows down viral replication.

Resistance of Herpesviruses

The resistance of herpesviruses is low or moderate. When dried upon inanimate surfaces, these agents survive from several hours to 7 days (cytomegaloviruses), or from few hours to weeks (herpes simplex viruses). HSV-2 is more sensitive than HSV-1.

Epstein-Barr virus is the most labile pathogen; it can't be isolated from environmental objects due to the rapid loss of viability.

Herpesviruses are readily inactivated by pH<4, UV irradiation or sunlight, and by heating (30 min at 60-80°C).

Viruses are sensitive to most disinfectants (e.g., sodium hypochlorite, povidone-iodine and other halides, phenol, aldehydes, ethanol, isopropanol, and others).

HERPES VIRAL INFECTIONS IN HUMANS

Infections, Caused by Herpes Simplex Viruses of Types 1 and 2

Special features of herpes simplex viruses

There are two distinct herpes simplex viruses: type 1 and type 2 (***HSV-1***, ***HSV-2***). Their genomes exhibit substantial homology. These two viruses demonstrate serological cross-reactivity.

Herpes simplex viruses express at least 11 structural ***glycoproteins*** with versatile biological functions (from gB to gM).

Glycoproteins ***gD*** and ***gB*** are the viral ***receptors***, responsible for adherence and viral entry. Glycoproteins ***gD*** and ***gB*** also stimulate the production of virus-neutralizing antibodies.

Several proteins impact host immune responses – protein ***gC*** is a complement-binding factor, and ***gE*** acts as Fc receptor for human IgG.

Glycoprotein ***gG*** is serotype-specific antigen allowing discrimination between HSV-1 (***gG-1***) and HSV-2 (***gG-2***).

The HSV growth cycle proceeds rapidly being completed in 8-16 h.

Pathogenesis, clinical findings and immunity

Infection with herpes simplex viruses is common among human population.

The viruses propagate rapidly being ***highly cytolytic***.

HSV-1 is usually associated with oropharyngeal lesions and causes recurrent attacks of ***herpes labialis*** or ***"fever blisters"***.

HSV-2 primarily infects the genital mucosal tissues resulting in ***genital herpes***.

Total spectrum of herpes simplex-associated diseases ranges from local gingivostomatitis and conjunctivitis to severe genital disease, encephalitis, and generalized infections of newborns and immunocompromised adults.

Herpes simplex viruses produce latent infection in neural tissues with periodical exacerbations. Recurrences of infections are common.

HSVs are ***transmitted*** through mucosal surfaces or skin lesions (intact skin is not permeable for virus).

HSV-1 is spread by ***airborne*** (***aerosole***) route or by ***direct contact*** with infected saliva. In most of cases the infection locally affects the oropharynx.

HSV-2 is usually transmitted by ***sexual intercourse*** like other kinds of ***sexually transmitted diseases***.

Neonatal herpes occurs after intra- or postnatal infections with either HSV-1 or HSV-2 (***vertical transmission***).

Viral replication primarily occurs at the site of entry. Within infected cells HSV inhibits the expression of HLA-I class molecules, thereby impairing presentation of viral Ags and making difficult elimination of infected cells by immune system.

Further HSVs invade local nerve endings and undergo retrograde axonal transport to dorsal root ganglia, where after several replications ***latency*** is established.

Oropharyngeal ***HSV-1*** infections result in viral persistence in the *trigeminal ganglia*, whereas genital ***HSV-2*** persists within infected *sacral ganglia*.

Herpes simplex viruses stay latent within infected ganglia ***lifelong***.

Various ***triggering stimuli*** like axonal injury, UV irradiation, fever, stresses and many other challenges induce viral replication. The mechanisms of viral activation are not well-elucidated yet.

The viruses move along axons to their peripheral sites within the skin or mucous membranes, where viral replication reoccurs.

Many of HSV-1 infections are asymptomatic. Symptomatic diseases demonstrate short incubation period (about 3-5 days), and clinical manifestations last for 2-3 weeks.

Gingivitis is the most common lesion in infants, primary infections in adults result in pharyngitis or tonsillitis.

Recurrent HSV-1 infections are commonly manifested as *cold sores* (***fever blisters***) near the lips.

Primary eye HSV-1 infection leads to severe ***keratoconjunctivitis***.

Genital disease is usually caused by HSV-2. This clinical variant is characterized by vesicular and ulcerative lesions of genitalia, which are very painful. Viral discharge lasts near 3 weeks.

Relapses of genital herpes are common but milder.

Herpetic encephalitis is seldom but life-threatening form of herpes simplex infections.

The most severe ***generalized forms*** of herpetic infections develop in immunocompromised patients, resulting from HSV dissemination.

Neonatal herpes demonstrates variable clinical manifestations, but in many cases it progresses into systemic infection with viremia and viral encephalitis. HSV-2 causes the most severe infections.

Cell-mediated ***immunity*** (e.g., T cytotoxic and natural killer cells) as well as host interferon responses are pivotal for efficient control of primary and recurrent HSV infections.

During primary herpetic infection short-term IgM antibodies are next followed by IgG and IgA antibodies that stay for a long period. Specific antibodies don't abolish reactivation of a latent virus but may reduce disease manifestations.

Laboratory diagnosis

The ***specimens for examination*** are taken from viral herpetic lesions; throat washings and cerebrospinal fluid can be used as well.

PCR assays are most commonly used for viral identification.

Viral cultivation is performed in primary tissue cultures. HSVs are further identified by immunofluorescence or neutralization tests.

The diagnostic value of ***serological testing*** is limited. Elevation of specific antibodies in 4-7 days after primary infection with a peak in 2-4 weeks is detected by ELISA.

Principles of treatment and prophylaxis of herpesviral infections

A number of efficient antiviral agents is used for ***treatment*** of HSV infections. Among them are ***acyclovir*** and ***valacyclovir***. Both inhibit viral DNA synthesis. Topical ***acyclovir*** applications are effective in herpes labialis.

However, antivirals don’t influence on latent HSVs that stay within sensory ganglia.

Experimental vaccines of various types are being developed. Nevertheless, there is no efficient HSV vaccine in current clinical practice.

Varicella-zoster Herpesvirus Infections

Special features of varicella-zoster virus (***VZV***)

Human herpesvirus ***type 3*** or varicella-zoster agent is a typical herpesvirus.

The same virus causes ***chickenpox*** (***varicella***) and ***zoster*** (***shingles***). Viral isolates from the patients with these diseases exhibit no significant genetic variation.

Chickenpox develops in case of ***exogenous*** VZV infection, whereas *shingles* emerges after reactivation of ***endogenous*** latent virus.

VZV propagates in cultures of human embryonic cell lines and produces chracteristic intranuclear inclusions.

Pathogenesis, clinical findings and immunity of VZV infections

Varicella is a primary VZV infection.

Communicability of varicella is very high. It is solely ***anthroponotic*** disease. Almost all unvaccinated humans acquire the infection.

Varicella-zoster infections are ***transmitted*** via ***airborne*** (***aerosole***) route. The ***sources of infections*** are sick persons with varicella or zoster disease.

The portal of viral entry is the mucosal epithelium of upper respiratory tract or eye conjunctiva.

The incubation period of ***varicella*** lasts for 10-21 days.

The virus circulates in blood resulting in viremia, undergoes multiple cycles of replication, and eventually localizes in the skin. Viral infection induces the formation of giant multinucleated cells with characteristic ***nuclear inclusions***.

Malaise and fever are the earliest symptoms, followed by next ***vesicular rashes*** appearance. It arises upon whole body surfaces on the skin of face, trunk, limbs, buccal mucosa, etc.

Varicella complications are rare. The virus may affect inner organs and brain with encephalitis. Immunocompromised patients are at increased risk of various complications of varicella.

Primary VZV infection is believed to confer ***lifelong immunity*** to varicella.

After the disease viral ***latency*** is established followed by VZV persistency within vertebral and cranial nerve ganglia.

Exacerbations of ***herpes zoster infection*** or ***shingles*** are stipulated by reactivation of dormant VZVs. The disease is triggered by various factors, which are not well-defined: body cold, UV irradiation, stresses, injuries, flare-up of systemic diseases, etc.

The disease usually occurs in adults over 50 years of age.

It is considered that insufficient immune surveillance permits reemergence of viral replication in ganglia, causing intensive inflammation of dorsal roots and pain. Virus migrates down the nerves to the skin and mucosa and induces vesicle formation.

The skin lesions of ***zoster*** are the same as in varicella but much more painful. The disease onset is followed by severe pain in the area of innervation of affected sensory nerves and ganglia. After exacerbation a chain of vesicles arises over the skin along the afflicted nerve. The most common complication of zoster is ***postherpetic neuralgia***. The pain may last for weeks or months. It is typical for ophthalmic zoster.

Zoster can emerge in the presence of relatively high levels of neutralizing antibody to varicella. Thus, ***cell-mediated immunity*** is regarded as the most important defense barrier in combat against varicella-zoster infection.

The repeated attacks of HZV infections are rare in immunocompetent individuals.

Overall, ***immunocompromised*** persons are the most predilected for zoster infections. Recurrent shingles is a common ***AIDS-indicator disease***.

Laboratory diagnosis

In most cases the diagnosis of varicella and shingles is established on clinical grounds.

Laboratory testing confirms the viral origin of diseases.

Microscopy of smears of scrapings or swabs from the vesicles reveals multinucleated giant cells with ***nuclear inclusions***. These are absent in nonherpetic vesicles.

Varicella-zoster specific ***antigens*** are determined by ***immunofluorescence*** method or ***ELISA***.

Viral DNA can be detected in vesicle fluid, in extracts of crusts, or in biopsy materials by ***PCR***.

Virus isolation is performed from vesicle fluid in embryonal human cell cultures within 3-7 days. Viral identification is made by immunofluorescence, neutralization tests and PCR.

Serological examination detects elevation of specific antibodies in the patient's serum mainly by ***ELISA*** test.

Principles of treatment and prophylaxis of VZV infections

Varicella is usually a mild disease and requires ***no treatment***, except complicated forms of infection in immunocompromised patients.

Several antiviral agents are effective against VZV including acyclovir, valacyclovir, famcyclovir, and interferons.

An efficient ***live attenuated varicella vaccine*** is available now for ***specific prophylaxis*** of the disease. It greatly reduces the number of infection cases and confers stable and long-lasting immunity.

Infections, Caused by Epstein-Barr virus

Special features of Epstein-Barr virus

The structure of ***Epstein-Barr virus*** (***EBV***, or human herpesvirus ***type 4***) is generally similar to other herpesviruses but with certain specific morphological traits.

EBV genome harbors about 100 genes encoding three groups of viral antigens: *latent phase antigens*, which maintain the state of viral latency; *early antigens* – nonstructural proteins, required to promote productive viral replication, and *late antigens*, which are the structural components of viral capsid (*capsid antigens*) and viral envelope (*membrane antigens*).

As the result, EBV life cycle may result in productive infection with ***lytic replication*** or come to ***latency***.

Specific viral antigens ***VCA*** (viral capsid antigen) and ***EBNA*** (EBV nuclear antigen) are worthy for diagnosis of infection.

EBV has only two target cells for replication – human ***lymphocytes*** (primarily, ***B cells***) and epithelial cells.

By means of supercapsid glycoproteins EBV binds to specific membrane receptors of B lymphocytes – first to ***CD21*** (or receptor for the C3d component of complement) and then to B-cell ***HLA class II*** molecules. This stimulates virus entry by endocytosis.

Epithelial cells capture EBV via membrane integrin receptors instead of CD21.

Upon infection of human B cells, EBV triggers their polyclonal activation with proliferation and blast transformation.

After active replication the virus converts to latency. Within small portion of memory B cells EBV persists lifelong.

EBV latency is maintained by presence of viral DNAs as circular episomes within infected B cells. It is considered, that some part of viral DNA might be integrated with human genome.

EBV demonstrates marked ***oncogenic potential*** especially for lymphoid cells.

Pathogenesis, clinical findings and immunity

Epstein-Barr virus is a ubiquitous herpesvirus that is the causative agent of ***acute infectious mononucleosis***, ***nasopharyngeal carcinoma***, ***Burkitt's lymphoma***, and some other lymphoproliferative disorders (***lymphomas***).

Infectious mononucleosis is a typical acute EBV infection. It has subclinical or mild course in children before the age of 10 and manifested disease in adolescents and young adults.

Incubation period usually lasts for about 30-50 days.

The infection affects humans only. The disease is mainly ***transmitted*** by ***infected saliva*** (e.g., by kissing) and initiates infection in the oropharynx. The virus invades B lymphocytes and epithelial cells of pharynx and salivary glands and disseminates throughout the body.

EBV activates growth program of B cells resulting in their polyclonal ***blast transformation*** and intensive proliferation followed by active replication of virus. Infected blasting B cells are recognized and massively destroyed by activated $CD8^+$ cytotoxic T cells (T killers) that finally curb the infection.

As the result, some part of viruses switches lytic cycle to latency and stays in memory B cells (and, probably, epitheliocytes) until the reactivation of virus occurs.

Clinical illness is characterized by the triad of symptoms – fever, sore throat and lymphadenopathy. Enlargement of cervical lymph nodes, spleno- and hepatomegaly are the characteristic findings in the disease.

There is a substantial increase in the number of circulating white blood cells, represented by ***atypical mononuclears***. Most of them are activated $CD8^+$ cytotoxic T cells.

The typical ***illness is self-limited*** and lasts 2-4 weeks. After clinical recovery asymptomatic individuals may shed the virus for many months.

Reactivations of EBV latent infections can occur but usually not manifested.

Severe recurrent infections may develop in immunosuppressed patients, e.g., after allogeneic transplantation.

Burkitt's lymphoma (a tumor of the jaw in African children and young adults) is considered to be associated with Epstein-Barr virus. Most of these tumors in Africa (> 90%) contain EBV DNA and viral antigens.

It is supposed that EBV virus may be involved at an early stage in Burkitt's lymphoma by immortalizing B cells. Malaria is regarded as cofactor that promotes EBV-induced cell transformation. Finally, specific chromosome translocations appear that affect immunoglobulin genes and deregulate *c-myc* proto-oncogene expression.

Nasopharyngeal carcinoma is the malignant tumor of epithelial cells that predominantly occurs in males of Chinese origin. EB virus DNA is commonly detected in nasopharyngeal carcinoma cells, and the patients demonstrate high levels of antibody to EBV. The tumor is poorly differentiated and aggressive, being infiltrated with lymphocytes.

Finally, Epstein-Barr virus is supposed to participate in pathogenesis of other ***lymphoproliferative disorders***, such as ***Hodgkin's disease*** (i.e., lymphogranulomatosis), and some B cell ***lymphomas***.

EBV infections elicit an intensive ***immune response*** both antibody- and cell-mediated. IgG antibodies against VCA and EBNA viral antigens circulate lifelong. However, cell-mediated reactions remain crucial for the control of infection. Active synthesis of interferons by affected cells facilitates viral clearance.

Laboratory diagnosis of EBV infections

Every case of EBV infection (e.g., infectious mononucleosis) needs laboratory confirmations.

PCR is the method of choice to detect EBV nucleic acid in ***clinical specimens*** (saliva, blood, or biopsies of lymphoid tissues).

Viral antigens can be detected in patient's samples by ***immunofluorescence***.

EBV isolation is rarely performed due to the difficulties of viral culture.

Serological testing is commonly used in clinical practice. The detection of IgM anti-VCA antibodies confirms acute EBV infection.

Principles of infection treatment and prophylaxis

Prophylaxis is ***non-specific***. Various kinds of experimental vaccines are being developed now.

The ***treatment*** of infectious mononucleosis is symptomatic. Valacyclovir demonstrates some clinical benefit in cases of manifested disease.

Cytomegalovirus Infections

Special features of cytomegalovirus

Cytomegalovirus (***CMV***, human herpesvirus ***type 5***) is also commonly spread in human population.

Nowadays CMV agent is a global ***leading cause of congenital infections***, followed by childhood hearing loss and neurodevelopmental delay with mental retardation.

Cytomegaloviruses exhibit a number of characteristic traits:

– viruses are strictly adapted to human host; CMV propagates only in human cells;

– CMVs contain the largest genome of human herpesviruses with marked genetic variability;

– viruses demonstrate slow replication rate; the length of viral replication cycle is over 70 h, this leads to long incubation period of CMV infection;

– high variety of clinical manifestations affecting various organs and tissues with special emphasis to CNS;

– generally low pathogenicity for immunocompetent individuals;

– propagating within immune cells, CMVs suppress cell-mediated immune response;

– lifelong persistency with repeated subclinical reactivations;

– evident teratogenicity resulting in high rate of congenital abnormalities;

– CMV produces characteristic cytopathic effect in cell cultures – the affected cells become enlarged, nuclear cytoplasmic inclusions appear.

Pathogenesis, clinical findings and immunity in CMV infections

CMV infection is largely a social health problem. In developing countries and resource-limited communities the contraction of the infection occurs in early childhood primarily due to infected breast milk feeding, poor living conditions (overcrowdness) and generally low attention of health authorities to CMV burden.

In industrialized countries the prevalence of CMV infection in women of childbearing age exceeds 50%, whereas in developing countries it usually above 90%.

Three main clinical groups of infections are caused by CMV:

– ***congenital disorders*** in newborns and infants;

– ***generalized*** (***systemic***) viral infections and ***CMV pneumonia*** in ***immunocompromised*** individuals (e.g., in ***AIDS*** patients);

– CMV infections in ***graft recipients after organ transplantation***.

Humans are the only known hosts and ***sources of infection*** for cytomegalovirus.

Airborne and ***oral transmissions*** are the most common in cytomegalovirus spread.

It can also be transmitted ***vertically*** from mother to child, by ***contact*** route via contaminated fomites, by ***organ transplantation***, via blood transfusion, or by sexual intercourse.

CMV infection in immunocompetent adults is usually mild or subclinical disease.

Incubation period lasts about 30-40 days. Manifested disease reveals mononucleosis-like syndrome with viremia, fever, lymphocytosis, and moderate hepatitis.

The virus is capable of invading virtually any type of host cells – endotheliocytes, leukocytes, epithelial cells, fibroblasts, parenchymatous cells of inner organs. CMV ***inhibits apoptosis*** of infected cells maintaining viral survival. During infection CMV actively sheds with saliva, genital secretions and urine, breast milk.

After 2-4 weeks the symptoms of the disease decline, as the virus is actively eliminated by reactions of humoral and cell-mediated immunity, and CMV comes into state of ***latency***.

Latent infection is ***lifelong***. The virus persists in $CD14^+$ monocytes and bone marrow progenitors. The genome of virus stays as episome within infected cells.

Reactivations of virus may be common but host immune response withstands viral propagation, preventing infection recurrence. Reactions of ***cell-mediated immunity*** are mandatory for efficient protection.

Congenital and ***perinatal*** CMV infections are characterized by CMV transmission across placenta in pregnancy, during delivery, and with infected breast milk after birth.

Cytomegalic disease of newborns often demonstrates systemic severe course with encephalitis and CNS damage. Lethality may achieve 30%.

The most common complications are sensorineural hearing loss (deafness), ocular abnormalities, and infant neurodevelopmental delay with mental retardation.

Severe generalized CMV infections develop ***in AIDS*** (AIDS ***indicator*** disease). They affect at least 15% of AIDS patients. CMV accounts for death of 10-20% of individuals with AIDS.

Finally, CMV produces life-threatening infections ***after organ transplantations***, being one of the substantial causes of death of graft recipients.

Laboratory diagnosis of CMV infections

Laboratory confirmation of CMV infection is of great value due to its non-specific clinical manifestations.

Specimens of throat washings, urine, saliva, blood, or autopsy materials are taken for examination.

For ***rapid cytomegalovirus detection*** immunofluorescence assay, ELISA and PCR are used.

PCR is the most reliable laboratory test in routine clinical practice for CMV identification.

Virus isolation is performed in human embryonated cell lines (e.g. fibroblast culture). In 1-2 weeks swollen cells with large intranuclear ***inclusion bodies*** (known as "***owl's eye***") are detected under microscopy.

Rapid identification of CMV isolates in cell cultures is made by immunofluorescence and PCR.

In ***serological testings*** specific anti-CMV antibodies are detected by ***ELISA***. Detection of antibodies of ***IgM*** class indicates ***primary*** CMV infection.

Principles of treatment and prophylaxis of CMV infection

Ganciclovir, a nucleoside structurally related to acyclovir, is used successfully to treat life-threatening cytomegaloviral infections in immunosuppressed patients. Donor's high-titer CMV immune globulin can be administered for pregnant women with active CMV infection.

Specific prophylaxis by vaccination is still ***not available*** for prevention of cytomegalovirus infection. Several candidate vaccines undergo clinical trials.

Non-speciic prophylaxis includes the maintenance of high personal hygienic conditions. Isolation of newborns with systemic CMV from other infants helps to prevent infection spread. Screening for cytomegalovirus infection is mandatory for graft donors and recipients.

Herpesviruses of Types 6, 7, and 8 in Human Pathology

T-lymphotropic ***human herpesvirus type 6*** (***HHV-6***) comprising two closely related viral species 6A and 6B is ubiquitous in human population. After the decline of protective maternal antibodies it rapidly infects infants – more than 90% of children above the age of 1 year and adults are seropositive for antiviral antibodies.

Despite the virus can infect a broad range of human cells, the main targets for HHV-6 infection are activated $CD4^+$ T lymphocytes.

Primary acute infection of HHV-6 affects children of 6 months to 3 years of age. Viral transmission occurs predominantly via oral secretions (saliva).

Typical febrile disease is known as *exanthema subitum* (or *roseola infantum*) that is followed by fever and skin rashes. The disease is self-limited.

It is generally ascertained now that HHV-6 may account at least for 10-20% of all febrile illnesses at this age.

After primary infection the virus comes into latency and persists lifelong in macrophages, bone marrow progenitors or CNS cells. The unique feature of latent HHV-6 genome is to make covalent linkages with host chromosomes.

Reactivations of virus are almost totally asymptomatic. Severe recurrent HHV-6 infections may develop after allogeneic transplantation in graft recipients.

Similarly, T-lymphotropic ***human herpesvirus type*** 7 (***HHV-7***) also appears to be a ubiquitous viral agent, which most infections arise in childhood. Persistent infections are established in salivary glands; the virus can be isolated from saliva of infected individuals.

The distinct relations between HHV-7 and human disorders remain to be established. HHV-7 as well as HHV-6 may have concern to the development of human chronic fatigue syndrome.

Last human herpesvirus, called ***Kaposi's sarcoma-associated herpesvirus*** (***KSHV***) or ***human herpesvirus type 8***, was first detected in 1994 in Kaposi's sarcoma biopsies of AIDS patients.

This virus is lymphotropic. Viral replication is very slow. It influences cellular genetic elements responsible for cell proliferation and host immune response (cytokine production, chemokine receptor expression, etc.) Human herpesvirus 8 seems to account for Kaposi's sarcoma, vascular tumor of mixed cellular origin in AIDS patients.

KSHV is not as ubiquitous as other herpesviruses, affecting about 5-10% of human population. It might be sexually transmitted among homosexual men, e.g. following HIV infection. Also the virus can be transmitted via solid organ transplantations.

Chapter 20
CAUSATIVE AGENTS OF ZOONOTIC VIRAL INFECTIONS

ARTHROPOD-BORNE AND RODENT-BORNE VIRUSES: GENERAL CHARACTERISTICS

Most of zoonotic viruses that may affect humans are the members of 2 ecological groups – *arboviruses* and *roboviruses*.

Arboviruses (or ***ar****thropod-****bo****rne* ***viruses***) comprise a broad ecological group of viruses ***transmitted*** to animal and human hosts ***by arthropod vectors***.

Similarly, ***rodent-borne viruses*** (or ***robo****viruses*) fall into ecological group of zoonotic viruses ***transmitted from rodents*** to susceptible humans.

That kind of division is not related with any viral taxonomic category. These groups embrace highly diverse viral representatives but sharing the number of common characteristics of their natural circulation and spread.

Numerous arbo- and roboviruses pertain to a vast line of viral families namely *Arenaviridae*, *Bunyaviridae*, *Togaviridae*, *Filoviridae*, *Flaviviridae*, and some others.

Many of them cause the severest human infections manifested by two clinical syndromes – viral ***fevers*** and viral ***encephalitis***.

Most clinically relevant representatives of arthropod-borne and rodent-borne viruses are listed in Table 10.

Table 10

Main arthropod-borne and rodent-borne viruses that cause human diseases

Viral genera	Viral representatives	Clinical disease	Infection source or vector	Basic properties
Family *Bunyaviridae*				
Genus ***Hantavirus***	Hantaan virus Sin Nombre virus	Hemorrhagic fever with renal syndrome Hantavirus pulmonarysyndrome	Rodent borne	ss (–) RNA segmented Helical or icosahedral Enveloped
Genus ***Nairovirus***	Crimean-Congo hemorrhagic fever virus	Hemorrhagic fever	Arthropod borne: ticks	

Genus ***Phlebovirus***	Rift Valley fever virus Sandfly fever Naples virus	Rift Valley fever Sandfly fever	Arthropod borne: mosquitoes, ticks, sandflies	
Genus ***Orthobunyavirus***	California encephalitis virus	Encephalitis, fever	Arthropod borne: mosquitoes	
Family *Togaviridae*				
Genus ***Alphavirus***	Eastern and western equine encephalitis viruses Venezuelan equine encephalitis virus Chikungunya virus, O'nyong-nyong virus, and many others	Encephalitis Hemorrhagic fevers	Arthropod borne: mosquitoes	ss (+) RNA Icosahedral Enveloped
Family *Flaviviridae*				
Genus ***Flavivirus***	Tick-borne encephalitis virus St. Louis encephalitis virus Japanese encephalitis virus Murray Valley encephalitis virus Yellow fever virus Dengue virus West Nile virus Omsk hemorrhagic fever virus Zika virus	Encephalitis Hemorrhagic fevers Putative teratogenicity	Arthropod borne: mosquitoes, ticks	ss (+) RNA Icosahedral Enveloped
Family *Arenaviridae*				
Genus ***Mammarenavirus***	Lymphocytic choriomeningitis virus. Lassa virus Guanarito, Junin, Machupo, Sabia, Lujo viruses	Fever, meningitis Hemorrhagic fevers	Rodent borne	ss (–) RNA segmented, ambisense Enveloped

Family *Filoviridae*				
Genus ***Ebolavirus***	Zaire ebolavirus Sudan ebolavirus Bundibugyo ebolavirus Taп Forest ebolavirus	Hemorrhagic fevers	Presumptively bat- or rodent-borne	ss (–) RNA Helical Enveloped
Genus ***Marburgvirus***	Marburg virus	Hemorrhagic fever		

The note: single-stranded nucleic acid is designated as **ss**.

Certain viral human pathogens from arthropod-borne and rodent-borne ecological groups are discussed below.

FLAVIVIRUSES: TICK-BORNE ENCEPHALITIS VIRUS

The History of Virus Discovery

Tick-borne encephalitis virus (***TBEV***) was discovered in 1937 by Soviet virologists L. Zilber, M. Chumakov, and E. Levkovich during their Far East expedition.

Classification

Tick-borne encephalitis virus (***TBEV***) pertains to *Flaviviridae* family and genus *Flavivirus*. The genus encompasses 53 zoonotic viral species that pertain to *arboviruses,* contracted by arthropod vectors to animal and human hosts.

Structure of Virus

TBEV is a middle- or small size virus (20-40 nm) of ***spherical*** shapes with lipid ***envelope***. TBE virion has ***icosahedral symmetry***.

Viral genome consists of ***positive single-stranded non-segmented RNA***.

Viral particle has a RNA-bound nucleocapsid ***C*** protein and envelope proteins ***M*** and ***E***

External E glycoproteins make viral spikes with receptor functions, promoting viral binding to the cells. They possess ***hemagglutinating*** activity.

Genomic RNA encodes a number of non-structural proteins (***NSP***) with enzymatic activity (*helicase*, *protease*, *RNA polymerase*).

TBE virus has 3 distinct genetic subtypes: European, Siberian, and Far Eastern.

All TBE viruses form similar common antigenic complex.

Virion Resistance

TBE virus as all flaviviruses is moderately resistant to the external influences. Nevertheless, it remains long stable at low temperatures. Virus maintains viability at least for 10 days at 16-18^{o}C.

Within contaminated dairy products (milk) it may stay viable for 2 months.

Heating at 60^{o}C for 10 min and boiling for 2 min irreversibly inactivates virus.

Virus is sensitive to UV light and all conventional disinfectants (halides, aldehydes, phenol, H_2O_2, detergents, ethanol, etc.)

Viral Replication Cycle

TBEV infects various types of cells, including leukocytes, endotheliocytes, hepatocytes, and neurons.

The virus attachs to the host membrane receptors by spikes and enter the cells by ***endocytosis***. Viral uncoating is facilitated with endosome acidification resulting in fusion of lipid envelope with endosomal membrane. Viral nuclecapsid passes into ***cytoplasm***, where uncoating and further replication occurs.

Genomic (+) RNA translation results in primary *polyprotein* synthesis that further processed by viral and cellular proteases into mature viral proteins.

Newly formed viral RNA polymerase enzyme activates genomic RNA replication. Viral genome is transcribed through intermediate minus RNA strand that serves as the template for final positive sense RNA synthesis.

Virion assembly and maturation is followed by egress of progeny viruses that leave the cell by ***budding***. Active viral propagation results in destruction of infected cells.

Virus is ***easily cultured*** in embryonated chicken eggs, various continuous (Vero and BHK cells) or primary cell lines, and in experimental animals (mice) under intracerebral inoculation.

Pathogenesis, Clinical Findings and Immunity in Tick-Borne Encephalitis

Tick-borne encephalitis is ***endemic zoonotic*** disease registered in the areas of habitations of tick vectors. The broad endemic regions of infection cover Far East, Siberia, Urals, Central Asia, Northern, Central and Eastern Europe. The disease cases were indicated from France to Japan. The largest number of cases is regularly reported from Russian Federation – 5,000 to 13,000 annually.

Each of genetic subtypes of viruses – European, Siberian, and Far Eastern – prevails in its corresponding geographical areas.

Belarus is also an endemic country for TBE caused by European subtype of virus. Above 110 cases of the disease were registered in 2014.

The ***sources of infection*** are the numerous species of wild and domestic animals (primarily, ***rodents***, hares, ungulates, predators, birds and many others, totally about 130 species).

The infection has a predominant ***vector-borne route of transmission*** via the ***bites of infected ticks***.

The main vector for Siberian and Far Eastern virus subtypes is tick species *Ixodes persulcatus*, for European subtype – *Ixodes ricinus*.

According to local data, about 15% of tick vectors are infected by TBEV. Tick vectors have the transovarial transmission of virus

Rare ***alimentary*** transmission was occasionally registered in past via contaminated non-pasteurized goat or cow milk.

The disease is characterized with *spring-summer* ***seasonality***.

Incubation period of tick-borne encephalitis averages 1-2 weeks.

According to seroprevalence studies, it has been established that about 90% of cases may be subclinical or asymptomatic.

After tick bite the virus enters the blood and lymph. It may primarily propagate in the site of bite and infect leukocytes and endotheliocytes. Hematogenous and lymphogenous dissemination spreads the pathogen throughout the body; the virus appears in spleen and liver and finally reachs CNS.

Viral propagation causes ***degeneration of neurons*** in spinal cord and brain followed by meningeal damage. The most suffering are the ***motor neurons*** of brainstem, cervical and upper thoracic parts of spinal cord.

In case of manifested TBE disease the ***onset is sharp*** with fever, headache and vomit.

Meningeal symptoms, sensory and coordination disorders and muscular pareses are observed. In severe cases the patients develop acute flaccid ***paralysis*** that predominantly affects the muscles of neck, shoulders, and upper limbs. The muscular weakness and paralysis may stay long after the end of acute disease course. Progression of the disease may be fatal.

The most severe are the clinical cases caused by Far Eastern subtype of virus. They demonstrate lethality of 20-40% and the high rate of poorly resolving neurologic sequelae.

The disease, caused by European virus subtype, has a milder course usually followed by complete recovery with lethality less than 1-2%.

Post-infectious ***immunity*** is long-term and stable. Virus-neutralizing antibodies maintain their protecting levels for a long time.

Laboratory Diagnosis of TBE

Clinical ***specimens*** of patient's blood, serum, cerebrospinal fluid or autopsy materials are taken for examination.

For ***rapid detection*** of virus immunofluorescence assay, ELISA and RT-PCR are elaborated.

Serological testing is actively used in routine laboratory practice. Anti-TBEV IgM antibodies are determined in patient's sera by ELISA indicating acute disease. The elevation of specific antibodies in the course of infection is determined in paired sera tests by hemagglutination inhibition assay, ELISA, or neutralization tests in the cell cultures.

Virus isolation is made by inoculation of filtrates of virus-containing specimens into various cell lines. TBE virus is detected in cell cultures by immunofluorescence test and PCR.

Animal experimental infection is performed by intracerebral inoculation of mice followed by animal postmortem examination with identification of virus (immunofluorescence assay, ELISA, or PCR).

Infectivity of ticks is determined by immunofluorescence and ELISA.

Prophylaxis and Treatment of Tick-borne Encephalitis

Specific antiviral ***treatment*** of tick-borne encephalitis is not elaborated. As the result, the treatment is largely symptomatic (infusion and detoxication therapy, neurological support). Passive protection with antiviral immune globuline might be worthy at early course of the disease.

Specific prophylaxis is performed by ***vaccination*** with ***inactivated*** cell culture-derived TBE vaccines.

The persons from the professional groups of risk of tick exposure that work in the endemic areas are vaccinated (e.g., forest workers, hunters, laboratory personnel, operating with infectious materials, etc.) In hyperendemic areas mass vacination of population should be conducted.

Post-exposure prophylaxis of individuals subjected to infected tick attack is performed by specific antiviral immune globuline being efficient within 2-3 days after the exposure.

Non-specific prophylaxis is primarily based on tick control measures achieved by broad use of chemical acaricidal agents in endemic areas. This substantially reduces the total number of living vectors, and therefore, the risk of infection.

Personal protection against tick bites should be maintained as well.

FILOVIRUSES: CAUSATIVE AGENTS OF EBOLA HEMORRHAGIC FEVER

The History of Ebolavirus Discovery and Current Epidemiological Situation

Severe hemorrhagic fevers caused by filoviruses (Lat. *filum* – thread) are the endemic diseases of West and Equatorial Africa, where they were met long ago.

First filovirus was isolated in 1967 in Marburg (Germany) by R. Siegert and coworkers after the laboratory outbreak of hemorrhagic fever in Yugoslavia and Germany. Primarily infected laboratory personnel have operated with monkey cell cultures, delivered from Uganda (Africa). From 31 affected people 7 died.

The isolated agent was designated as Marburg virus and the disease was referred to as “Marburg hemorrhagic fever”.

Subsequently the limited outbreaks of the disease were repeatedly registered in Africa, the last occured in 2014.

In 1976 a great outbreak of a new hemorrhagic fever emerged in Sudan and Zaire that involved more than 600 people with 430 fatality cases. Outbreak onset was located in Yambuku village near the Ebola River.

Initially presumed Marburg virus as the causitive agent of epidemic was further rejected after the thorough study of a novel isolated pathogen termed as ebolavirus (S. Pattyn and coworkers, Belgium, 1976; K. Johnson and coworkers, USA, 1976).

In 1982 both Marburg and Ebola agents were placed into newly formed viral family *Filoviridae*. Later it has become known that Ebola disease is caused by closely related several species of ebolaviruses.

Since 1975 the outbreaks of Ebola hemorrhagic fever were regularly registered in Central and West Africa.

In December 2013 a new epidemic of Ebola virus disease emerged in Guinea; the infection rapidly spread to the neighbouring countries (Liberia, Sierra Leone and others) and moved outside the initial region. The single cases of infection were diagnosed in United States, European countries and in a number of African states).

Due to its serious community health threat in 2014 WHO constituted Ebola disease outbreak as Public Health Emergency of International Concern (PHEIC).

To April 2016, more than 28,800 cases of Ebola virus disease were indicated (above 15,000 of them were laboratory confirmed) that resulted in 11,325 death cases. Therefore, outbreak lethality reached almost 40%.

Only after intensive united efforts of international organizations and national state authorities the Ebola epidemic was terminated. Last cases of the disease were officially registered in March, 2016.

On March 29, 2016, WHO ended the state of the Public Health Emergency of International Concern for the Ebola outbreak in West Africa.

Classification of Filoviruses

The family *Filoviridae* pertain to the order *Mononegavirales*.

The causative agents of ***Ebola virus disease*** (***EVD***) and ***Marburg virus disease*** (hemorrhagic fevers) pertain to genera *Ebolavirus* and *Marburgvirus*, respectively. Genetic similarity between two genera is moderate – less than 50%.

Marburgvirus genus has a single viral species Marburg virus.

Genus *Ebolavirus* harbors 5 closely related viral species Zaire ebolavirus, Sudan ebolavirus, Bundibugyo ebolavirus, Тап Forest ebolavirus, and Reston ebolavirus, where first four species cause hemorrhagic fevers in humans. The most severe disease is related with ***Zaire ebolavirus***.

Structure of Ebolaviruses

All ebolaviruses contain linear ***single-stranded negative-sense non-segmented RNA***. The viruses look like long cylindrical ***thread-like*** structures about 80 nm in breadth and 600-1000 nm in length.

These viruses are covered with the external lipid ***envelope***. Viral nucleocapsid displays the ***helical*** symmetry.

Ebolaviruses contain nucleocapsid proteins bound to viral RNA. Internal protein ***L*** develops ***RNA polymerase*** activity.

GP glycoproteins are the outer structural components of the viral envelope. During reproduction, GP proteins are largely produced in soluble form (*sGP*).

The ***matrix viral proteins*** (VP) support envelope structure

Virion Resistance

The viability of ebolaviruses is moderate. Within aerosol droplets they retain infectivity at least for 1-2 hours, when dried – for several days. Sunlight and UV radiation readily inactivate them.

These viruses can withstand low-temperature exposure. Dried blood spots with ebolaviruses remain viable at 4^{o}C for 3-4 weeks.

Heating at 60^{o}C for 30-60 min and boiling for 5 min irreversibly inactivates virus.

Viruses are sensitive to most of conventional disinfectants (halides, aldehydes, phenol, detergents, ethanol, etc.) For ebolaviruses WHO recommends surface disinfection with household bleach (sodium hypochlorite).

Viral Replication Cycle

The target cells for ebolavirus replication are the all cells of monocyte/macrophage lines, dendritic cells, endotheliocytes, adrenal cells, hepatocytes.

Viral attachment to the host cells is mediated via ***GP*** proteins binding to numerous membrane receptors (*lectins* and many others)

Specific binding induces viral penetration by ***endocytosis***. Acidification of endosome facilitates viral uncoating and RNA release.

The replication of ebolaviruses occurs in the ***cytoplasm*** of infected cells.

At first viral mRNAs are transcribed on genomic (–) RNA matrix. mRNAs are next translated on cellular ribosomes resulting in viral proteins.

New genomic (–) RNAs are reproduced by viral RNA polymerase via the step of (+) RNA intermediate that serves as the template for progeny genome synthesis.

Nucleocapsid assembly is performed at the inner part of cytoplasmic membrane. Maturating virions migrate across the cell membrane, where they are covered with lipid envelope. Finally ebolaviruses are released from the infected cells by ***budding***.

Filoviruses are characterized with high reproduction rate. Massive egress of nascent virions results in destruction of infected cells.

Culture of ebolaviruses is performed only in specially organized and highly equipped national centers (laboratories) that maintain biocontainment precautions at ***biosafety level 4*** (***BSL-4***) as the ***highest level of biosafety*** precautions.

BSL-4 is created for rapidly transmitted microbial pathogens, which cause diseases with highest fatality rate (like ebolaviruses).

In BSL-4 conditions ebolaviruses can be readily cultured within continuous cell lines (Vero cells or others), or by inoculation in laboratory animals (guinea pigs, hamsters, mice, or primates).

Pathogenesis, Clinical Findings and Immunity in Ebola Virus Disease

Hemorrhagic Marburg and Ebola fevers are extremely dangerous, highly contagious and fatal ***zoonotic*** viral infections.

Both agents stay in WHO Risk Group 4 pathogens and US list of "Biological Select Agents or Toxins" being present in Tier 1 of this list (the highest rank of public threat).

The ***sources of infection*** in cases of Ebola disease outbreaks are ***sick animals*** (primates, swine, or certain species of antelopes) or ***sick humans***, primarily taking care on patients with Ebola.

Fruit bats and less likely rodents seem to be the animal reservoirs that harbor ebolaviruses in natural conditions.

The infection is ***transmitted*** predominantly via ***direct*** or ***indirect contact*** of human susceptible host with ***infected excretions*** of sick animals or humans.

Also the disease is contracted by ***alimentary route*** after the ingestion of infected meat.

High concentration of viruses is observed in patient's blood, and in sufficient amounts in feces, vomits, breast milk, or sperm that is enough for transmission. In this vein Ebola infection can be transmitted by sexual intercourse still over 3 months after clinical recovery.

Aerosol spread of infection is only possible in cases of great concentration of virus in droplets. Direct air droplet human-to-human transmission of Ebola disease is not registered.

The virus is not transmitted by arthropod vectors.

Medical workers treating patients with Ebola, as well as other caregiving persons are at the highest risk of infection. In Africa the infection spread is maintained by traditional burial rituals with unprotected contacts with the deceased.

Incubation period varies from 2 to 21 days (an average of 6-10 days).

The causative agent usually enters the body through skin lesions or cuts. Also it can penetrate conjunctiva. The virus propagates in regional lymph nodes. Hematogenous and lymphogenous dissemination spreads ebolaviruses throughout the body; they appear in all inner organs and tissues.

The main targets for ebolaviruses are the ***cells of innate immunity*** (monocytes, macrophages, dendritic cells, neutrophils), endothelial cells, and the cells of parenchymatous organs.

Extensive viral reproduction results in massive cell death, activates systemic inflammation with parallel deep suppression of antiviral immune responses.

The viruses activate ***apoptosis*** of lymphocytes, monocytes and macrophages.

Soluble ***sGP*** protein arrests neutrophil activation. Structural ***VP*** proteins inhibit the secretion of both types of interferons.

In 2-3 days the viruses affect vascular endothelium all over the body's tissues resulting in ***generalized vasculitis***.

Hard damage of hepatocytes and endothelial cells leads to disseminated intravascular coagulation (***DIC***) that entails hypotension and collapse. As the result of systemic infection, hemorrhagic and necrotic lesions emerge in all organs and tissues. This is followed by massive internal bleedings and tissue edema resulting in ***hypovolemic shock*** with possible fatal outcome.

The infection has acute onset with fever above 38^{o}C and extensive pain syndrome (headaches, abdominal and chest pain, muscular and joint pain). At 5-7 days of the disease about 50% of patients exhibit skin rash, followed by internal bleedings and mucosal hemorrhages.

Hematemesis, hemoptisis, and post-injection bleedings can be observed.

The development of hemorrhages and hypovolemic shock seriously worsenes the prognosis of Ebola virus disease.

The lethality of Ebola outbreaks is very high – it varies from 20 to 90% (average at 50%).

The recovery is slow, the convalescents produce long time shedding of virus. Hearing and vision disorders are common.

Post-infectious *humoral* ***immunity*** renders the high levels of specific antiviral antibodies but their role in protection against reinfections remains unclear.

Due to their prominent virulence, rapid and severe disease course, and high fatality of infection, ebolaviruses are generally ascertained as the potential agents of bioterrorism and biological warfare.

Laboratory Diagnosis of Ebola Virus Disease

Taking into account an extreme danger of Ebola infection all manipulations with ebolavirus agents should be performed in laboratories maintaining biosafety level 4 (BSL-4) as the highest grade of biocontainment precautions.

The ***specimens*** are taken from patient's ***blood*** and autopsy materials

Laboratory diagnosis of infection is verified by ***RT-PCR*** and other ***molecular genetic tests*** detecting viral nucleic acids.

Viral antigen is determined by ***immunochromatography***.

Isolation of ebolaviruses is not routinely used because of high demands to biosafety. The virus is cultured in various cell lines with its further identification by PCR.

For ***serological diagnosis*** ELISA test is elaborated detecting antiviral IgM and IgG antibodies.

Principles of Prophylaxis and Treatment of Ebola Virus Disease

A substantial threat of Ebola virus disease requires strict measures in order to prevent the emergence and spread of the infection. It needs to keep professional and public awareness, heightened infection surveillance with rapid case validations, patient isolation and management. Additional measures should be directed to control travellers visiting the areas of disease outbreaks.

Vaccines for ***specific prophylaxis*** of Ebola infection will be soon introduced into clinical practice as several genetically engineered vaccines from Russia, USA, and Great Britain manufacturers are starting clinical trials now.

There is still no specific ***antiviral treatment*** for Ebola disease. Positive result is achived by administration of the sera of convalescent patients with high titers of specific antiviral antibodies.

Extensive supportive treatment greatly amends the disease prognosis. The prevention of hypovolemic shock and DIC development, the maintenance of vital body functions including active fluid resuscitation with correction of electrolyte disbalance and coagulation disorders favor the outcome of Ebola infection.

ARENAVIRUSES: CAUSATIVE AGENT OF LASSA HEMORRHAGIC FEVER

The History of Arenavirus Discovery

First arenavirus – a causative agent of lymphocytic choriomeningitis – was isolated in 1933 by R. Lilly and C. Armstrong in USA.

Further it has been discovered that most of arenaviruses are the severest ***zoonotic pathogens*** that in some situations may cause acute ***hemorrhagic fevers*** in humans with high fatality rate. These infections are endemic in various regions of Africa and Latin America.

Arenavirus known as Junнn virus, a causative agent of Argentine hemorrhagic fever was discovered in 1958 by A. Parodi; Machupo virus, the agent of Bolivian hemorrhagic fever was found by K. Johnson in 1963; Lassa virus, the agent of Lassa hemorrhagic fever was isolated in Nigeria by J. Frame in 1969; Guanarito virus of Venezuelan hemorrhagic fever – by R. Salas in 1991; and Lujo virus of hemorrhagic fever – by W.I. Lipkin in Africa in 2008.

Classification of Arenaviruses

The viral members of family *Arenaviridae*, pathogenic for mammals, are assigned to genus *Mammarenavirus*.

The most dangerous for humans are numerous species – the agents of viral ***hemorrhagic fevers*** like Lassa virus or Junнn, Machupo, Guanarito, Lujo, and some other less common viruses.

According to their geografical location these pathogens are divided into the Old World viruses and the New World viruses.

Lassa virus is responsible for most of endemic cases of arenaviral hemorrhagic fevers.

Besides these agents, the virus of lymphocytic choriomeningitis may also cause human disorders (e.g., fever or meningoencephalitis). Also it demonstrates evident teratogenic potential.

The name of *arena*viruses originated from Lat. *arena* – sand, as these agents hold in their envelope sand-like granular inclusions – captured cellular ribosoms – visible by electron microscopy.

Structure of Arenaviruses

Arenaviruses are polymorphic ***enveloped*** viral particles 50-300 nm in size.

Genome of arenaviruses comprises ***2 segments*** (small ***S*** and large ***L***) of linear ***single-stranded ambisense*** (–) ***RNA***.

The ambisense nature of genomic RNA indicates that synthesis of viral proteins is based on either *genomic* or its *complementary antigenomic* RNA templates.

Every segment of viral genomic RNA codes for 2 viral proteins.

L segment is responsible for synthesis of ***L*** protein (*RNA polymerase*) and *matrix **Z*** protein.

S segment encodes ***NP*** nucleoprotein and ***GP*** glycoprotein of viral spikes. The molecule of ***GP*** is further proteolyzed into ***GP1*** (*receptor* protein) and ***GP2*** (*fusion* protein).

Nucleoprotein ***NP*** and *matrix* ***Z*** protein inhibit the synthesis of interferons by infected cells, thus promoting viral dissemination.

Virion Resistance

Outside the host arenaviruses stay alive 15-30 minutes to several hours; their survival increases at low humidity conditions.

The viruses are sensitive to UV radiation and sunlight.

Heating at 60^{o}C for 30-60 min irreversibly destroys arenaviruses.

They are readily inactivated by all conventional disinfectants.

Viral Replication Cycle

Viral attachment to host cells is promoted by binding of receptor ***GP1*** protein to various membrane molecules (e.g., transferrin receptor) present on many types of human cells.

Specific binding induces viral ***endocytosis***. Acidification of endosome activates ***GP2*** protein that stimulates uncoating and viral entry into cytoplasm with genomic RNA release.

The replication of arenaviruses is performed in the ***cytoplasm*** of infected cells.

After synthesis of viral genomic RNAs by viral RNA polymerase and translation of viral proteins capsid assembly is performed at the inner part of cytoplasmic membrane. Viral self-assemblage is mediated by matrix Z protein. Occasionally viral particles may capture cellular ribosomes.

Virions are released through the cell membrane, being covered with lipid envelope. Viral egress occurs by ***budding***.

Culture of arenaviruses similar to isolation of other causative agents of acute hemorrhagic fevers (e.g., filoviruses) is performed only in reference centers (laboratories) that operate at highest ***biosafety level 4*** (***BSL-4***).

In BSL-4 conditions arenaviruses can be isolated in various cell lines (e.g., Vero cells), or by inoculation into laboratory animals (guinea pigs, or primates).

Pathogenesis and Clinical Findings of Lassa Hemorrhagic Fever

From all arenaviral diseases ***Lassa hemorrhagic fever*** seems to be the most life-threatening infection. Being localized in endemic regions of West Africa (e.g., in Nigeria, Liberia, Guinea and some other countries) it affects up to 300,000 people annually resulting in a number of human deaths about 5,000-10,000.

The incidence of other arenaviral hemorrhagic fevers (e.g., Junнn, Machupo, Guanarito, or Lujo diseases) is substantially lower.

Lassa virus belongs to the arenaviruses of the Old World.

Lassa hemorrhagic fever is a typical ***zoonotic*** endemic disease. It pertains to ecological group of ***rodent-borne*** infections.

The main ***source of infection*** is a certain species of endemic African ***rodent*** (Natal multimammate rate or *Mastomys natalensis*). These animals continuously excrete the viruses with feces and urine.

The mechanisms of development of Lassa hemorrhagic fever share a number of common traits with other hemorrhagic fever diseases (e.g., caused by filoviruses).

The infection is ***transmitted*** predominantly by ***contact*** route; ***foodborne*** and dust ***aerosol*** transmission are also possible.

Infectious dose for Lassa hemorrhagic fever is ***extremely low*** – it is estimated as 1-10 viral particles.

Incubation period lasts 1-2 weeks.

Despite the high virulence of the infectious agent, more than 80% of infection cases are mild or subclinical, indicating the natural resistance of local human communities.

Lassa virus usually enters the host through the mucosal or skin lesions. The virus propagates in regional lymph nodes. Hematogenous dissemination results in ***viremia***. The levels of viremia correspond to disease severety.

The main targets for Lassa viruses are the ***cells of innate immunity*** (primarily, monocytes, macrophages, and dendritic cells).

The basic mechanism of the development of systemic viral infection is the prominent ability of arenaviruses to inhibit cellular and humoral reactions of antiviral immunity.

Viral nucleoprotein ***NP*** and matrix ***Z*** protein inhibit the synthesis of interferons and activation of macrophages and dendritic cells. This blocks the presentation of viral antigens to immune lymphocytes and sharply diminishes cytokine secretion.

In conditions of immune insufficiency arenaviruses rapidly spread throughout the body, affecting parenchymatous organs and other tissues.

The viruses affect endothelial cells with deep microcirculation disorders.

Lassa disease manifests with fever and ulcerative pharingitis, followed by cough and vomiting. It progresses towards ***generalized hemorrhagic syndrome*** resulting in ***multiple organ dysfunctions*** with severe hepatitis, myocarditis, viral pneumonia, encephalitis, and renal failure.

The lethality in hospitalized patients is about 15-20%; amongst pregnant women – more than 80%. Hearing loss is a common neurologic sequela.

Convalescence is slow; the virus sheds for 1-3 months after the recovery.

Laboratory Diagnosis of Arenaviral Infections

All manipulations with Lassa viruses and other arenaviral agents are elaborated in laboratories of biosafety level 4 (BSL-4).

The ***specimens*** are taken from patient's blood, urine, sputum, cerebrospinal fluid, nasopharyngeal washes, and autopsy materials.

The virus is most rapidly identified by ***RT-PCR*** or other ***molecular genetic tests***.

For detection of viral antigens ***immunofluorescence*** or ***ELISA*** tests are elaborated.

Isolation of arenaviruses is conducted in various cell lines or by inoculation into laboratory animals followed by viral identification with ELISA or PCR.

For ***serological diagnosis*** ELISA test is elaborated detecting specific antiviral antibodies of IgM class.

Principles of Prophylaxis and Treatment of Arenaviral Infections

Antiviral drug ***ribavirin*** is commonly used for ***treatment*** of arenaviral hemorrhagic fevers. If administrated on the 1st week of the disease, it substantially reduces lethality.

Administration of the sera of convalescent patients with high titers of specific antiviral antibodies facilitates the recovery.

Other treatments presume the support of vital body functions of respiratory and cardiovascular system.

Common ***prophylaxis*** measures are ***non-specific*** and similar to other cases of hemorrhagic fevers.

Various kinds of vaccines for prevention of Lassa fever are actively developed now. A first example of efficient live arenaviral vaccine against Argentine hemorrhagic fever caused by Junнn virus has been introduced already into clinical practice.

RABDOVIRUSES – RABIES VIRUS

The History of Virus Discovery

Rabies disease is well-known since antiquity. It is a zoonotic acute infection of central nervous system that is inevitably fatal.

First breaking success in the fight against rabies was achieved in 1884 by Louis Pasteur and his outstanding colleagues Emile Roux, Charles Chamberland, and Louis Thuillier, who created efficient antirabies vaccine. Since that time this mortal disease began to retreat.

V. Babes in 1887 and A. Negri in 1903 described specific inclusion bodies in neurons of animals, dead from rabies. These inclusions were referred to as ***Babes-Negri bodies***.

The viral etiology of rabies was proved in 1903 by P. Remlinger, E. Riffat-Bay, and A. di Vestea, who isolated rabies virus.

Although the number of human cases is small, rabies is a major public health problem because it is broadly spread among animal reservoirs.

Classification of Rabies Virus

This virus pertains to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*, and species rabies virus.

To date 7 genotypes and 5 serotypes of rabies virus are established. Overall, lyssaviruses demonstrate the lowest genetic variability in comparison with other rhabdoviruses.

Structure of Virus

Rabies viruses are the rod- or ***bullet***-shaped particles of about 75x180 nm. Type of symmetry of virions is ***helical***.

Viruses are surrounded by lipid ***envelope*** with protruding spikes. Ribonucleocapsid is confined inside the envelope. Viral genome includes ***single-stranded***, non-segmented ***negative-sense RNA***.

Genomic RNA encodes 5 structural proteins.

Nucleocapsid proteins ***N*** (nucleoprotein), ***P*** (phosphoprotein), and ***L*** (RNA polymerase) account for replication of viral genome and viral mRNA transcription.

Matrix ***M*** protein is located under lipid envelope; it takes active part in viral budding.

Supercapsid ***glycoprotein G*** makes spikes in lipid envelope. It plays an essential role in rabies pathogenesis.

First, it specifically binds to ***nicotinic acetylcholine receptors*** (***nAChR***) on membranes of neurons and muscle cells promoting viral attachment and membrane fusion. Also protein G stimulates ***apoptosis*** of infected cells.

Furthermore, superficially located protein G activates host immune response in the course of infection. It elicits the synthesis of virus-neutralizing antibodies.

According to structural variations of G proteins, rabies virus is divided into 5 serotypes. However, all viral serotypes are enough similar and induce the formation of ***cross-reactive neutralizing antibodies***. Thus, it became possible to use only ***1 serotype*** of vaccine virus for rabies vaccination.

When freshly isolated in the laboratory from external source, the viral strain is designated as rabies ***street virus***. These viruses show long and variable incubation periods (usually 21-60 days in dogs) and regularly produce cytoplasmic inclusion bodies. Sequential brain-to-brain passages in rabbits primarily made by L. Pasteur yielded a "***fixed***" virus. This pathogen lost the ability to multiply in extraneural tissues. Fixed mutant virus propagates rapidly, and its incubation period has been shortened to 4-6 days. Inclusion bodies are found rarely in this infection.

Virion Resistance

Rhabdoviruses demonstrate generally low resistance. Rabies virus is inactivated rapidly by exposure to ultraviolet radiation or sunlight, and by heating (1 hour at 50°C or 1 minute at 100°C).,

Nevertheless, it remains long-time infectious at low temperatures, e.g. it stays viable at 4°C for weeks.

Rabies virus is sensitive to the commonly used biocides (e.g., sodium hypochlorite and other halides, detergents, aldehydes, ethanol, ether, and others). The virus loses viability at pH<3 or pH>10.

Viral Replication Cycle

Rabies virus attaches to cells via its glycoprotein spikes. Spike ***G protein*** binds to nicotinic ***acetylcholine*** receptor (***nAChR***) facilitaing viral entry by ***endocytosis***. Molecules of ***nAChR*** are expressed on the membranes of neurons and muscle cells, so these cells are the primary targets for virus.

Acidification of endosome content activates G proteins. It results in envelope-membrane fusion and viral penetration into cytoplasm followed by uncoating.

Rabies virus replication occurs in ***cytoplasm*** of infected cells.

Single-stranded RNA genome is transcribed by virion-associated RNA polymerase ***L*** to mRNA. Messenger RNAs code for five structural virion proteins: nucleoprotein (*N*), polymerase (*L*), phosphoprotein (*P*), matrix (*M*), and receptor glycoprotein (*G*).

Negative-sense genomic RNA is transcribed via complementary positive-sense RNA intermediate. Newly synthesized genomic RNAs associate with N, L, and P proteins with formation of ribonucleocapsids. They further interact with matrix M proteins in cytoplasm.

The nascent virions acquire an envelope and external spikes, when released by ***budding*** through the cell plasma membrane, where G proteins were primarily embedded.

Rabies virus is ***readily cultured*** in brain tissues of laboratory mice, syrian hamsters, or rabbits; the infected animals display encephalitis with paralysis.

Also the virus is adapted to various cell lines (Vero cells, BHK cultures and others). Acidophylic inclusions (***Babes-Negri bodies***) are detected in cytoplasm of infected cells.

Cytopathic effect of rabies virus is not observed.

Pathogenesis, Clinical Findings and Immunity in Rabies

Rabies is acute ***zoonotic neuroinfection*** developing after the bite of a rabid animal and followed by progressive CNS damage with ***lethal encephalitis***.

Without urgent vaccination rabies disease demonstrates ***100%*** fatality.

Rabies virus has a broad host range. All warm-blooded animals, including mammals, can be infected.

Susceptibility of many mammalian species is very high (e.g. foxes, wolves, racoons and raccoon dogs, cats, rats and many others.)

Recovery from infection in animals is extremely rare except certain bats species. For instance, vampire bats may transmit the virus for months without any signs of disease.

Thus, the ***source of infection*** is a *rabid animal*.

The virus is usually ***transmitted*** to humans via the ***rabid animal bite*** or by ***contact with infected saliva*** through the skin or mucosal lesions.

Incubation period varies strongly depending on host's immune status, the amount of inoculum, and the distance the virus should move from the point of inoculation to the central nervous system. The shortest incubation period (about ***7-10 days***) is observed in patients bitten on the face, head or neck; the longest occurs in cases of bites on feet (***1-3 months***). Moreover, some documented cases of rabies had the incubation period of more than 10 years. Thus, rabies is regarded as *slow viral infection*.

When entered into the tissues, rabies virus binds to nicotinic ***acetylcholine*** receptor (***nAChR***) on the membranes of neurons and neuromuscular junctions.

Next pathogenesis of infection follows two basic ways.

If the virus locally multiplies in muscle tissue at the site of inoculation, it may stay long and propagate in primary location up to 2 months.

By contrast, if the virus binds to neuronal transport proteins *dynein* or *neurotropin* in axoplasm of peripheral nerves, it undergoes fast retrograde axonal transport that delivers virus directly to bodies of neurons in spinal cord and brain. The velocity of viral movement is equal to 50-100 mm/daily that substantially shortens the incubation period.

Next the virus multiplies in the nuclei of CNS in spinal cord, brainstem, hippocampus, thalamus, cerebellum and other CNS parts.

The progeny viruses spread further through peripheral nerves to the salivary glands and other tissues. The highest concentration of rabies virus is observed in submaxillary salivary gland. Viruses are also found in skeletal muscles, retina and cornea, heart, kidneys and other inner organs. However, rabies virus is not isolated from patient's blood.

The virus produces characteristic eosinophilic cytoplasmic ***inclusions***, ***Babes-Negri bodies***, within infected neurons. This finding is pathognomonic on rabies.

The disease manifests as acute fulminant ***fatal encephalitis***.

During the acute neurologic phase the patients demonstrate deep neurologic disorders. Patients exhibit *hydrophobia* (fear of water), *photophobia* (fear of light), *aerophobia*, noise phobia. They feel a profound thirst; their swallowing is impaired. Severe sympathetic hyperactivity results in hypersalivation, increased perspiration and lacrimation. The patients often show aggressiveness.

In case of paralytic forms of infection initial local paralysis progresses into generalized disease.

The late phase of rabies is followed by coma and death, usually 2-7 days after the disease onset. The major cause of death is respiratory paralysis.

Laboratory Diagnosis of Rabies

All the animals indicated as "rabid or suspected rabid" should be sacrificed immediately for laboratory examination of nervous tissue. Other animals should be observed for 10 days. If they demonstrate any signs of encephalitis, or unusual behavior, they should be killed and the neural tissues examined.

Post-mortem diagnosis of rabies is highly specific.

The ***specimens*** of brain tissues taken from died animals or patients are used for laboratory diagnosis. The virus is most rapidly identified by ***immunofluorescence*** or immunoperoxidase staining using antirabies antibodies.

Cytological examination of slides reveals ***Babes-Negri bodies*** in the neurons of brain or spinal cord of affected person.

Reverse transcription-PCR can be used to detect rabies virus genome in brain tissue.

For ***viral isolation*** the tissue samples are inoculated intracerebrally into suckling mice. Infection in mice results in encephalitis and next death. Animal CNS tissues are examined for viral antigen or Babes-Negri bodies.

Similarly the virus can be inoculated into laboratory cell lines with further identification by immunofluorescence, cytology, ELISA or PCR.

Antemortem (***intravital***) ***diagnosis*** of rabies is applied in cases of atypical infections in humans and, more often, for rapid diagnosis of animal infection needs to urgent vaccination of affected individuals.

Tissue biopsies of bite sites and back of neck skin, buccal and corneal epithelium, cerebrospinal fluid or saliva are examined by immuno-fluorescence or PCR.

Detection of serum specific antibodies by ELISA might be helpful in monitoring of humoral response in vaccinated persons.

Principles of Rabies Prophylaxis and Treatment

There is still no treatment for clinical rabies.

Thus, ***post-exposure*** rabies ***prophylaxis*** is ***lifesaving intervention*** and should be initiated without any delay. The medications include rabies vaccination, administration of rabies immune globulin if required, and perfect surgical management of bite wounds.

If the vaccine or specific antibodies are timely administered, virus propagation arrests, and the virus can't invade the central nervous system. Passively administered antibodies lower the concentration of virus, providing additional time for a vaccine to stimulate active antibody production, thereby preventing viral entry to CNS.

All the vaccines for humans contain inactivated rabies virus. The most commonly administered is ***human diploid cell vaccine***.

Vaccine is injected 5-6 times into deltoid muscle. It confers long-term immunity, postvaccinal complications are rare.

Live attenuated vaccine can be used for animal vaccination.

For ***passive post-exposure prophylaxis*** specific immunoglobulins are administered.

Equine rabies immunoglobulin is obtained from horses hyperimmunized with rabies vaccine virus.

Human anti-rabies immunoglobulin is a globulin fraction prepared from the plasma of vaccinated humans. It renders fewer side effects in comparison with equine antibodies.

Passive prophylaxis is administered ***prior to vaccination*** in case of multiple bites on upper limbs, neck, or head, where ***the incubation period is short***.

Chapter 21
PRIONS AND PRION DISEASES

General Characteristics of Prion Agents

Prions are the novel unique class of infectious agents completely different from all microbial pathogens known before. The name "***prion***" abbreviates from words "***proteinaceous infectious particle****"*, thus emphasizing the ***protein nature*** and ***infectivity*** of these unusual pathogenic agents.

Prion diseases afflict humans as well as various animal species causing slow but severe progressing neurodegenerative disorders with characteristic spongioform degeneration in central nervous system (***transmissible spongiform encephalopathies*** or ***TSEs***).

The History of Discovery

As far as in 1939 the cases of strange disease of sheep marked by severe itching of animals were described. Thereafter, the disease was named as "***scrapie***", and its infectious nature was proven further by direct experimental infection of goats.

Later in the 1950s, C. Gajdusek and coworkers first demonstrated another strange neurodegenerative disorder spread among some tribes inhabiting New Guinea island. The disease, called ***kuru***, was transmitted throughout the population of the island by ritual cannibalism, being ultimately fatal.

Further the distinct similarities between above mentioned diseases and some other known human and animal disorders, e.g. sporadic form of ***Creutzfeldt-Jakob disease*** (***CJD***), or ***bovine spongiform encephalopathy*** (***BSE***) were noticed. In the early 1980s all these clinical data together with the number of evident laboratory findings allowed future Nobel Prize Laureate S. Prusiner to propound a ***prion hypothesis*** for explanation of origin of these diseases.

Owing to the discovery of prions made by S. Prusiner in 1982 it became obvious that not only DNA-containing agents can provoke diseases but the products of gene expression, proteins, are able to cause the disease emergence and progression. Prions differ from bacteria and viruses, as they are lack of any type of nucleic acids, DNA or RNA, being

solely of protein nature. Prions resist inactivation by procedures that modify polynucleotides, e.g. treatment with DNAse or RNAse. They are also extremely stable to heating.

Pathogenesis of Prion Diseases

Actual identification of prion molecules has become possible only after cloning of their specific gene ***PRNP*** (***PR***io***N P***rotein gene), located within 20th chromosome. This gene codes for normal cell glycoprotein ***PrP^{C}*** (the letter ***C*** in abbreviation means **c**ommon or **c**ellular). The gene is typically expressed in neurons and glial cells of CNS, as well as in leukocytes and some other cell elements. ***PrP^{C}*** molecule is supposed to play a role in signal transmission regulating synaptic activity. PRNP gene is evolutionary stable structure. It expresses not only in humans, but also in other mammalian species, birds, etc.

The infection arises after alteration of initial non-pathogenic form of PrP^{C} protein. The latter event ensues from PRNP gene mutations that are characteristic for hereditary prion diseases, e.g. ***Gerstmann-Straussler-Scheinker syndrome*** (***GSS***), ***fatal familial insomnia*** (***FFI***), or ***sporadic form*** of ***Creutzfeldt-Jakob disease***. Also the disease can start after acquisition of infectious prion molecules from external source. This is essential for epidemic ***bovine spongiform encephalopathy*** (***BSE***) or "*mad cow disease*" in cattle and suspected for emergence of a "***new variant***" of ***Creutzfeldt-Jakob disease*** in humans.

Pathogenic form of prion protein ***PrP^{Sc}*** (***Sc*** means scrapie) originates from initial PrP^{C} molecule after conformational change of its structure. Once appeared, pathogenic prion PrP^{Sc} spreads its abnormal conformation to surrounding intact PrP^{C} molecules as autocatalytic chain reaction.

It is well-determined that the animals, which are lack of PrP gene (for instance, experimental "knock-out" mice) are not affected by prions. They can't express pathogenic PrP^{Sc} molecules.

Generated prions are tough units, resistant to proteases, formaldehyde treatment and heating. They display distinct ability to self-aggregation forming amyloid protein deposits in brain tissue. This leads to the progression of neurodegenerative disorders of CNS.

General Manifestations of Prion Diseases

Prion diseases are generally characterized by severe non-inflammatory damage of neuronal tissue with spongioform degeneration and atrophy, neuronal loss, vacuolization, astrogliosis, progression of amyloid plaques.

Familial and ***sporadic*** (***hereditary***) forms of prion diseases in humans are rare (about 1 case per million of population in a year).

Infectious prion diseases are more common; in addition, some other neurodegenerative disorders of unknown origin are supposed now to be of prion nature.

Prion diseases that can afflict humans comprise a number of disorders. Among them are above mentioned ***Creutzfeldt-Jakob disease*** (sporadic, mixed and iatrogenic forms), ***Gerstmann-Straussler-Scheinker syndrome***, ***fatal familial insomnia***, and ***kuru*** disease.

Kuru and iatrogenic Creutzfeldt-Jakob disease are the ***infectious forms*** of human prion illnesses; the others are ***hereditary*** disorders. Human prion infections are contracted by alimentary route or by medical manipulations (e.g., after organ and tissue transplantations).

Every disease is characterized by some specific clinical and morphological traits. For instance, sporadic ***Creutzfeldt-Jakob disease*** gradually starts at the age of 50-60 years. First symptoms are not very specific; the patients demonstrate general asthenia, sleeplessness, headache, dizziness, and memory disorders. Progression of dementia is followed by motor dysfunction with cerebellar ataxia, myoclonic spasms, pyramidal and extrapyramidal disturbances, etc. The disease becomes fatal in one-two years.

Rare iatrogenic form of Creutzfeldt-Jakob disease usually occurs after organ transplantations, non-sterile medical manipulations, after cellular and tissue therapy, etc. Its incubation period lasts for more than 10 years

A great public interest to prion diseases was renewed after epidemic of ***bovine spongiform encephalopathy*** (***BSE***) or "*mad cow disease*" occured in Great Britain in 1986-1998. The disease affected about 200,000 cattle.

During the epidemic course more than 30 cases of a "***new variant***" of ***Creutzfeldt-Jakob disease*** were registered in humans. The onset of the disease developed young and adult persons 15-40 years old. It was strongly supposed that the illness contracted by alimentary route after ingestion of infected beef. Similar disorders were determined in animals (cats, zoo apes and others) kept with beef meet feeding.

Post-mortal examination of the patients died from "new variant" of Creutzfeldt-Jakob disease revealed alterations of brain tissue typical for spongiform encephalopathy in cattle.

Clinical findings are common with other prion diseases (psychotic reactions, dementia, cerebellar ataxia, myoclonic cramps, etc.) The insidious progression of CNS disorders leads to lethal outcome.

Kuru disease is only of historical interest now as the disease transmission is possible only by acts of ritual cannibalism that was eliminated among affected tribes long time ago.

Gerstmann-Straussler-Scheinker syndrome is a seldom disease. It is a familial or sporadic disorder that occurs mostly in the 4^{th}-5^{th} decade. Cerebellar ataxia and concomitant motion disturbances are common. The illness lasts several years to patient's death.

Fatal familial insomnia is inherited by autosomal dominant type. It is characterized by untreatable insomnia together with hyperthermia, tachycardia, progressing ataxia, myoclonic cramples, atrophy of the thalamus and other brain structures, memory loss. Mental disorders are followed by endocrine dysfunction with abnormal production of melatonin, prolactin, somatotropic hormone, etc. The disease predominantly afflicts young people.

Finally, a number of commonly spread diseases with still unknown pathogenesis show distinct similarity with prion disorders. For instance, prion hypothesis is relevant for ***Alzheimer's disease*** – widespread mental disorder of elderly that is followed by severe dementia. The same principle concerns ***Parkinson's disease*** and serum ***amyloidosis***.

The observed variations in the course and clinical manifestations of prion diseases are supposed to be dependent on existence of different strains of infectious prion molecules.

Laboratory Diagnosis of Prion Diseases

Laboratory confirmation of prion origin of the disease is the subject of great difficulties. It can be made by intracerebral inoculation of suckling mice or hamsters with postmortem material taken from the brain. The laboratory animals render some symptoms of prion infection in 150 days and even longer after primary inoculation.

Cytological examination of brain tissue of died animals reveals the injury of the brain tissue characteristic for prion disease (spongioform degeneration and atrophy, vacuolization, amyloid plaques, etc.).

Detection of prion molecules with highly ***specific monoclonal antibodies*** using various immunological tests (immunohistochemistry, western blotting, or ELISA) expanded the opportunities of diagnosis of prion infections. Nevertheless, conventional immunoassays are still less sensitive for diagnosis of minute amounts of prion molecules.

Quite recently ***ultra-sensitive prion assays*** based on highly sophisticated laboratory procedures and equipment were introduced into practice. They allow to detect negligible amounts of prions directly in clinical specimens, e.g. in blood of infected organisms (antemortem tests). Among them are immuno-quantitative PCR, protein misfolding cyclic amplification and the most novel SOFIA test (surround optical fiber immunoassay). The latter test is capable of detecting as low as 1 attogram (10^{-18} g) of prion substance.

Prophylaxis and Treatment of Prion Diseases

Non-specific prophylaxis is the only method known to date that is used for prevention of transmission of prion diseases. As the prion molecules show striking resistance to heating and chemical disinfectants the carcasses of dead animals should be burnt.

Efficient prophylaxis of iatrogenic form of Creutzfeldt-Jakob disease needs the tight control of medical sterilization and requires thorough selection of donor's tissues for transplantation.

Drug treatment of prion disorders is not yet elaborated. Promising new direction in this field implies the drug intervention into molecular pathogenesis of prion disorders, e.g. inhibition of remodeling of normal PrP^{C} into deleterious PrP^{Sc}.

Chapter 22
INFECTIOUS DISEASES WITH SPECIFIC LESIONS IN ORAL CAVITY
(***For students of Dentistry faculty***)

Tuberculosis

Tuberculosis is one of the most severe threats for human health at the beginning of XXI century. The current increase of new tuberculosis cases is about 2% per year, but the raise of *multidrug resistant* (*MDR*) *tuberculosis* and *extensively drug-resistant tuberculosis* (*XDR*) is much more rapid.

Therefore, the global spread of MDR tuberculosis is a problem of great medical and social importance.

The main sources of infection are persons with active tuberculosis.

Tuberculosis of oral cavity is a seldom clinical situation among common lesions of oral cavity. Nevertheless, every case of oral ulceration requires stringent differential diagnosis for tuberculosis.

Tuberculous ***oral injuries*** can be ***primary*** or ***secondary to pulmonary disease***; the latter are much more frequent.

Oral lesion in tuberculosis can resemble stellated ulcer. In many cases it affects the dorsum of tongue. The ulceration is usually painful.

Less often the lesions might be found on gingiva, lips, buccal mucosa, palate, or floor of mouth.

Laboratory diagnosis of disease is based on microscopy of specimen, taken from oral lesion, and isolation of *M. tuberculosis* on selective media. Ziehl-Neelsen acid-fast bacilli stain or fluorescent microscopy with auramine stain is used.

In most of cases oral tuberculosis is secondary to pulmonary disease, that's why sputum culture must be examined.

Specific treatment of tuberculosis grounds on long-term regimen of combined antimicrobial chemotherapy. It lasts up to 6 months. The list of first-line drugs comprises isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin. If necessary, oral mycobacterial lesions are subjected to surgical treatment.

Actinomycosis

The patients with ***actinomycosis*** of orofacial area comprise up to 80% of all infection cases. The main causative agents of the disease are *Actinomyces israelii* and *A. gerencseriae*. They are commonly associated with *Aggregatibacter actinomycetemcomitans* and propionibacteria.

In relatively large amounts actinomycetes reside in dental plaques of clinically healthy individuals. Also they are present in soil, contaminate herbs and grains. In this vein actinomycosis contraction occurs from *endogenous* or *exogenous* microbial infection.

The disease transmission is possible by airborne, contact or rarely by alimentary route. After initial contact with skin or oral mucosa the bacteria activate their lymphogenous or hematogenous spread into deep tissues. They can reach adipose tissue, muscles and fascia resulting in formation of long-term granulomatous and poorly healing abscesses. In case of progression the abscesses enlarge and may open spontaneously with purulent discharge.

Primary actinomycosis is readily complicated by secondary infection predominantly of anaerobic genesis.

Microbiological examination starts from microscopy of purulent discharges taken from inflammatory abscess lesions. Round-shaped branched microcolonies of actinomycetes are indicated. Their inner structure resembles mycelium – fungus-like branched network of hyphae.

Successful ***treatment*** of actinomycosis requires high-dose administration of antimicrobial drugs for which the bacteria retain sensitivity: beta-lactams, macrolides, or doxycycline.

Diphtheria

Diphtheria is the severe infectious disorder caused by toxigenic *Corynebacterium diphtheriae*.

Bacterial ***exotoxin*** plays the principal role in the pathogenesis of disease, blocking intracellular protein synthesis in tissues and organs.

Diphtheria is manifested by characteristic ***fibrinous inflammation*** with growing vascular permeability. It results in formation of tight “pseudomembranes” covering the tonsils, pharynx, or larynx. Proteinaceous pseudomembranes contain fibrin that is firmly attached to innermost tissues.

Diphtheria is anthroponotic disease. Patients with diphtheria and carriers are the main sources of infection. The disease is communicated by airborne route.

Exotoxin traverses the mucous membranes and causes the destruction of epithelium with inflammatory response and microcirculatory and coagulation disorders. The expanding necrotic pseudomembranes impede normal airflow. Any attempt to remove the pseudomembrane results in bleeding. Pseudomembrane respiratory obstruction (or *diphtheritic croup*) can cause patient suffocation. The regional neck lymph nodes enlarge and neck swelling progresses resulting in total neck edema ("bull neck").

Toxin absorption leads to distant toxic action with tissue damage, parenchymatous degeneration, fatty infiltration and necrosis in myocardium, liver, kidneys, and adrenals, sometimes accompanied by hemorrhages. The toxin also produces nerve damage often followed by paralysis of the soft palate, eye muscles, or limbs.

In dental practice the most common is the ***local form*** of diphtheria where pseudomembranes cover patient's tonsils.

Toxic and hypertoxic clinical forms are characterized with burst progression of the disease resulting in toxic shock that may cause patient death in two-three days.

Laboratory diagnosis of diphtheria depends on rapid determination of diphtherial exotoxin and isolation of toxigenic bacteria.

The presence of exotoxin in clinical specimen (primarily, in tonsillar pseudomembranes) is detected by enzyme-linked immunosorbent assay (ELISA test) or by immunoprecipitation. The toxigenicity of *C. diphtheriae* culture can be shown also by incorporation of bacteria into cell culture monolayers. Toxin diffuses into cells monolayer and causes cell destruction. Finally, PCR is used as the most sensitive, rapid and specific method for determination of gene encoding diphtheria toxin.

The treatment of diphtheria basically rests on the early administration of specific antitoxic antibodies that block toxin action.

Specific prophylaxis is afforded by active immunization. Usually combined DPT (or diphtheria-pertussis-tetanus) vaccine or combined tetanus-diphtheria toxoids are used. All the children must gain the repetitive course of diphtheria toxoid immunizations followed by several boosters in every 10 years.

Scarlet Fever

Scarlet fever is acute *streptococcal infection* caused by group A *Streptococcus pyogenes* that produce *pyrogenic exotoxins A, B and C*. The symptoms of the disease ensue from systemic toxin action. Toxins display superantigenic activity with massive production of proinflammatory cytokines. This results in fever, generalized rash, and skin desquamation. The disease profoundly impairs cardiovascular system especially microcirculation.

Scarlet fever transmission occurs by air-droplet route. The illness affects predominantly children. It begins from sharp raise of temperature, vomiting and throat pain. In 1-2 days characteristic skin rash appears and moves down from face to trunk and limbs. The patient demonstrates bright red cheeks and chin with a typical pale area around the mouth. The rash stays for several days and then gradually fades with skin desquamation (or peeling).

Almost all cases of scarlet fever are followed with specific oral lesions such as "strawberry" tongue with elevated deep-red lingual papillae and inflamed uvula.

Clinical diagnosis of the disease is supported by ***isolation of group A streptococci*** from patient's throat swab. Microbial culturing is performed on number of special media for streptococci (blood or serum agar).

Scarlet fever is efficiently ***treated with antibiotics***. Beta-lactams are the most commonly used antimicrobials here because of retained sensitivity of *S. pyogenes* to this group of drugs.

Anthrax

Anthrax is the severe zoonotic disease caused by toxigenic *B. anthracis*. Herbivores can become infected with anthrax by grazing in pastures that are contaminated with spores. Contact with animals (butchering, skinning, or exposure to hides or wool), and consumption of contaminated meat are the risk factors for infection in humans.

Depending on the site of the infection, anthrax cases display different clinical manifestations – cutaneous anthrax, inhalation anthrax (or wool-sorter's disease) and gastrointestinal disease.

In dental practice ***cutaneous anthrax*** may be observed. Here the spores of bacilli are introduced into the skin. Germination occurs within

hours, and vegetative cells produce anthrax toxin. The disease usually develops within 1-7 days after entry.

A red macule emerges at the site of inoculation. The lesion subsequently comes into a papular-vesicular stage that is followed by ulceration with a blackened necrotic ***eschar*** or *anthrax carbuncle* (malignant pustule) surrounded by brawny edema. This lesion is painless. A regional lymphadenitis is commonly seen in these patients. Eventually eschar dries, loosens, and separates; spontaneous healing occurs in 80 to 90% of untreated cases. However, in severe cases bacterial dissemination leads to septicemia with high mortality rate.

Anthrax diagnosis relies on clinical findings and disease epidemiology with subsequent microbiological confirmation. It includes microscopy of samples taken from primary skin lesions and bacterial isolation.

The patients are hospitalized and treated with antibiotics (fluoroquinolones or beta-lactams) and specific immunoglobulin.

Syphilis

Primary syphilitic lesions in oral cavity may inflict buccal and gingival mucosa, lips or perioral skin. They emerge in affected individuals after oral sex. The rates of other routes for disease transmission (via personal things such as tooth brushes, or by direct contact, or after medical manipulations) are negligibly low.

After sexual intercourse the causative agent of syphilis *T. pallidum* invades the skin or mucosals through their minimal lesions. Infectious dose for it is extremely low: as little as 1-5 microbial cells can trigger the illness.

Incubation period depends on inoculated dose. A large inoculum, e.g., about 10^7 bacterial cells, results in disease appearance in 5-7 days.

After approximately 1 month of incubation a ***hard chancre***, essential tissue lesion of *primary syphilis*, appears. It is followed by regional lymphadenopathy.

Chancre evolves at the primary site of microbial entry. Its orofacial localization often occurs on lip vermilions and oral mucosa. Hard chancre is a painless ulcer about 0.5-3 cm with sharp margins, clean base, induration, and sometimes with purulent discharge.

In most cases chancre heals spontaneously within about 6 weeks. Nevertheless, in several weeks the disease comes into stage of *secondary syphilis*, which results from lymphogenous and hematogenous microbial dissemination.

Secondary syphilis is the systemic inflammatory process characterized by skin rash, headaches, fever, malaise, lymphadenopathy, mucosal lesions, and CNS disorders. It lasts from 2-3 months to more than 1 year. Cutaneous or mucosal syphilitic eruptions or ***syphilides*** harbor great amount of spirochetes, being highly infectious.

In oral cavity secondary mucosal lesions appear as the erythematous and maculo-papular syphilides. They render oval-shaped grayish-white elements on mucosal surface followed by periostitis.

If not treated, after latent period of various length (about 1 year or even more) the disease progresses into *tertiary syphilis*.

Tertiary syphilis affects various body's organs and tissues, especially cardiovascular system and CNS. Specific slow-growing indurative injuries (or *gummas*) emerge in tissues and parenchimatous organs resulting in necrosis with subsequent connective tissue proliferation.

These lesions rarely appear in mouth. If arisen, they form growing nodules (tubercular syphilides) and gummas. When progressed, the lesions undergo deep necrosis with degradation of underlying soft tissues and bones, for instance, resulting in perforation of the soft palate.

Congenital syphilis in infants issues from vertical disease transmission in untreated women with a rate of 70 to 100% for primary syphilis.

The infected infants may be asymptomatic or show the numerous manifestations of early and late congenital disorder with multiple dental abnormalities. Herein they render screwdriver-shaped incisors with notched incisal edges (Hutchinson's incisors). Mulberry deformation of molars is observed.

Overall, *Hutchinson's triad* of abnormalities in congenital syphilis comprises lymphadenopathy and hepatomegaly accompanied with skeleton and teeth lesions.

Laboratory diagnosis of syphilis rests on microscopical examination of specimens taken from primary or secondary syphilitic lesions and/or serological tests for specific antibodies.

Serological testing is the mainstay of laboratory diagnosis for latent, secondary, and tertiary syphilis. It confirms the presence of anti-treponemal antibodies in patient sera by means of highly specific serological reactions, e.g., *T. pallidum* immobilization test and ELISA test.

The treatment of the disease is based on high sensitivity of *T. pallidum* to beta-lactam antibiotics. ***Penicillin G*** and its derivatives remain to be the drugs of choice for syphilis treatment.

Gonococcal Infection

Gonococcal stomatitis can arise in newborns infected from sick mothers in the course of delivery. In adults oral gonococcal infection usually follows urogenital infection in persons practicizing orogenital contacts.

Mucosal tissue, tonsils, pharynx and upper larynx are involved into inflammatory process. Inflammatory manifestations result in erythematous edema, suppurative erosions and ulcerations of oral mucosa.

Laboratory diagnosis of gonococcal infections is carried out by microscopy of the materials obtained from urogenital excretions and lesion discharges.

Antimicrobial ***treatment*** is performed by antibiotics with proven efficacy against gonococci because of high levels of resistance essential for these bacteria. Combinations of azithromycin with gentamycin or fluoroquinolones are preferentially used. Local treatment of specific oropharyngeal lesions with antiseptics can be applied.

Oral Candidiasis

Oral candidiasis (or ***candidal stomatitis***, oropharyngeal candidiasis and moniliasis) is the specific oral mycosis caused by yeast-like fungi of *Candida* genus.

More than 50% of disease cases is related with commonly spread fungi ***Candida albicans***; other causative species for candidiasis in descending rate are *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, or *C. krusei*.

C. albicans pertain to the normal representatives of oral microflora, where they can be found in modest amounts. However, in some specified conditions these fungal members exert serious *opportunistic infections* rendering local or severe systemic disease.

C. albicans are *dimorphic fungi*. In mouth they are often present as *yeast forms* or blastospores with questionable virulence whereas mould-like fungal *hyphal forms* are capable of invading host tissues. Transition between these two phases largely depends on changes of environmental conditions.

In addition candidae create *pseudohyphae* – long filaments composed of oval fungal cells that are closely attached on their poles. It occurs due to incomplete separation of cells after division.

Overall, all the details of transformation of oral saprophytic candida into aggressive fungal pathogens are not yet clear completely.

It is considered that candidal strains with enhanced capacity to adhesion and colonization are generally more pathogenic. In particular, the strains with high expression of certain adhesins (e.g., hyphal wall protein 1 or extracellular mannoprotein) are referred to as more active pathogens. Similarly, pathogenic fungi produce large amounts of hydrolytic enzymes such as proteases, phospholipases and hyaluronidase.

Oral candidiasis is regarded as the *most frequent opportunistic infection of oral cavity* that affects humans. Likewise, it is the most typical form of candidal infection and the most common form of oral mycosis.

Candidiasis affects newborns and infants, but most of all – ***inmunocompromised persons***, namely patients with HIV infection and acquired immunodeficiency syndrome (AIDS); patients with cancer under cytostatic chemotherapy; patients, treated with antibiotics of broad spectrum of action with deep oral or intestinal ***dysbiosis***.

Primary carriage of *C. albicans* predisposes to its further opportunistic infection.

Candidal infection is divided into acute and chronic, primary and secondary.

Primary oral candidiasis originates from resident fungi and affects oral cavity and perioral area. By contrast, *secondary* disease emerges after spread of disseminated candidal infection that occupies the mouth and other body compartments.

Nevertheless, dissemination of oral candidiasis to other body sites (or invasive infection) is generally seldom situation. It happens predominantly in immunocompromised patients.

There are 3 main forms of oral candidiasis:

1) pseudomembranous;

2) erythematous;

3) hyperplastic.

Pseudomembranous candidiasis, or *thrush*, demonstrates white pseudomembranous spots upon oral mucosa that contain epithelial and fungal cells mixed with fibrin and cellular decay. The covering film is easily removed showing red mucosal bottom. These lesions usually affect tongue, palate and buccal epithelium.

Chronic pseudomembranous candidiasis arises mainly in immunocompromised persons, e.g., HIV patients.

Erythematous form of the disease exposes typical smooth and reddened lesions, which are located mainly upon the tongue dorsum or

palate. The lesions are usually painful. Substantial loss of lingual papillae (or depapillation) is common here.

In most of cases this fungal infection is the result of long-term antibiotic treatment or corticosteroid therapy. That's why it is often named as "antibiotic induced stomatitis" or "antibiotic sore mouth".

Erythematous illness covers up to 60% of total oral candidiasis if taken with related clinical forms such as angular stomatitis and denture-related stomatitis. The latter is associated with chronic erythematous candidiasis.

Hyperplastic candidiasis is a seldom form of disease with the incidence of about 5%.

Candida-associated lesions are the oral injuries caused by candida fungi in association with other pathogenic microorganisms. Two basic forms are known – angular cheilitis and denture related stomatitis.

Angular cheilitis is the infection-based inflammation at mouth angles. About 20% of disease is caused solely by candidal species, whereas 60% originate from association of *C. albicans* and *S. aureus*. This pathology renders angular soreness and erythematous inflammation that may result in angular fissuring. The syndrome affects elderly adults and often accompanies denture related stomatitis

Denture related stomatitis is a low or moderate inflammatory complication that affects edentulous elderly adults wearing oral appliances (*dentures*).

It is generally ascertained that more than 50-60% of denture-wearing individuals exhibit denture related stomatitis.

Dominating causative agents in this pathology are candida fungi (above 90% of total number of cases). Thus the disease is commonly termed as "***Candida-associated denture induced stomatitis***" or CADIS.

Mucosal surface under dentures demonstrates highest grades of fungal colonization in comparison with normal unaffected mucosa. It creates acidic, moist and relatively anaerobic surrounding that promotes further candidal growth. In addition, poor adjustment of oral prostheses causes micro-injuries of gingival mucosa.

Candidae easily attach to the surface of damaged tissues and polymeric surface of dentures with their multiple pits and fissures. Microbial adherence and colonization stimulates local inflammation. This leads to continuous irritation of oral mucosa resulting in erythematous lesions.

Chronic denture related stomatitis creates the stable reservoir for candidal infection that in worsen conditions may expand to other areas of oral cavity.

Diagnosis of oral candidiasis grounds on clinical findings but requires laboratory confirmation of fungal infection.

Specimens are taken from lesion sites by oral smears, swabs or rinsing.

Microbial smears are examined by Gram stain demonstrating gram-positive candidal cells with pseudohyphae.

Fungal culture is made on *Sabouraud medium*.

Oral rinse examinations help to discriminate between "normal" candidal carriage and oral candidiasis. About 7,000-7,500 colony-forming units of candidae per 1 ml of oral rinse can be found in disease condition.

Treatment of oral candidiasis presumes administration of topical anti-fungal drugs, such as miconazole, nystatin, levorin, or amphotericin B.

Severely immunocompromised patients, e.g., with candidiasis in AIDS, need systemic anti-fungal therapy with oral or intravenous drugs (amphotericin B, azoles or others).

Proper oral hygiene strongly reduces candidal propagation in oral cavity. It presumes adequate toothbrushing, smoking cessation, oral rinsing after inhalation steroid use, and regular denture disinfection with denture cleaner preparations (e.g., chlorhexidine or sodium hypochlorite).

General Characteristics of Viral Infections, Affecting Oral Cavity

Viral lesions are considered among the most common in current dental practice.

Numerous viral infections cause specific alterations within oral cavity.

For instance, in severe influenza course hyperemia and cyanosis of oral mucosa are typical. Infectious mononucleosis is followed by tonsillitis with multiple *petechial rashes* that covers oral mucosa. Likewise, oropharyngeal lesions are common in parainfluenza infection, in rubella, rhinoviral and adenoviral diseases. Manifestations in viral hemorrhagic fevers comprise severe hemorrhagic rash, cheilitis, angular stomatitis, catarrhal gingivitis.

Herpes simplex virus, vesiculovirus and herpes zoster virus, Coxsackie A viruses, virus of vesicular stomatitis are able to cause infections with similar alterations in oral cavity. Primary lesion here is *vesicle* that is gradually changed into ulcerative erosion or *aphtha*.

Herpetic Oral Lesions: Herpes Simplex Infection

Viral infection, caused by 1st type of herpes simplex virus (***HSV-1***) is the most common viral disease in humans. By sensitive laboratory tests herpes simplex persistency is detected among 90% of adult population.

Infants in the age range from 6 month to 3 years are grossly susceptible to this infection. Large outbreaks of acute herpetic stomatitis emerge in child care settings.

The main clinical presentations of infection are *acute* (*primary*) *herpetic gingivostomatitis* and chronic *herpes labialis* (or *cold sores*) with its recurrent exacerbations.

Acute herpetic gingivostomatitis occurs as initial (primary) herpetic infection. It is caused by HSV-1 and in rare cases by HSV-2 that is common for genital infection.

Incubation period lasts for 4-5 days. It is followed by fever and appearance of characteristic lesions – pin-head vesicles, localized on lips, tongue and gingival or buccal mucosa. Soon the vesicles become eroded forming painful ulcerations.

Usually isolated viral lesions heal spontaneously in several days without scarring. Clinical recovery occurs in 1-3 weeks.

Nonetheless, in course of primary infection the virus invades local nerve endings and moves by retrograde axonal flow to dorsal root ganglia, where the *latency* is established.

Chronic ***herpes labialis*** (or *cold sores*) results from repetitive exacerbations of latent HSV-1 infection. In most of cases various exogenous stimuli (fever, UV irradiation, physical or emotional stress, axonal injury, etc.) activate viral replication. The virus moves along axons back to the peripheral site; and replication proceeds at the skin or mucous membranes. The vesicles commonly affect perioral area, lip vermilions and their borders, sometimes – soft and hard palate, tongue or buccal epithelium. Many recurrences are asymptomatic.

Clinical diagnosis of herpetic infection is confirmed with ***laboratory testing*** of specimens taken from herpetic lesions. Immunofluorescence analysis and PCR are the standard reactions in this condition. Serological testing states the elevated levels of specific antiviral antibodies of IgM class.

In mild or moderate cases acute herpetic gingivostomatitis doesn't require ***treatment***. Severe or complicated manifestations can be treated with topical applications of acyclovir on the background of adequate oral hygiene.

Herpangina

Herpangina (also known as *mouth blisters* or vesicular pharyngitis) is typically caused by ***Coxsackie group A*** viruses. In few cases it can be triggered by Coxsackie group B infection and echoviral infection. All these viruses are the members of *Enterovirus* genus and *Picornaviridae* family.

Coxsackie herpangina is a severe febrile pharyngitis. It affects predominantly babies and young children.

There is an abrupt onset of fever and sore throat with discrete vesicles on the posterior half of the palate, pharynx, tonsils, or tongue. The vesicles progress into ulcerations that heal spontaneously in 7-8 days.

The disorder is self-limited and resolves in 1-2 weeks.

Diagnosis rests on clinical findings; virus isolation is usually not required.

The treatment of herpangina is symptomatic; specific antiviral therapy is absent.

Oral Lesions in Measles

Measles is an example of extremely contagious acute respiratory viral infection. It is caused by specific *morbillivirus* of a single serotype. Measles is a human illness that usually affects children.

Virus replicates in respiratory tract epithelium and moves to regional lymphatic nodes (primary viremia). After second propagation in the lymphoid tissue it spreads throughout the body penetrating endothelium of vessels and epithelial cells in skin, conjunctiva, respiratory tract, and oral cavity.

Characteristic ***lesions of oral cavity*** in measles are known as ***Filatov's-Koplik's spots***. They appear upon the buccal mucosa two to three days earlier than the measles rash. The spots are the sites of intensive viral replication with formation of giant cells.

Koplik's spots can be discerned as firm white lesions on the buccal epithelium opposite to the lower 1^{st} and 2^{nd} molars. They are highly specific for the disease, sometimes described as "grains of salt on a wet background".

As Koplik's spots emerge at the beginning of measles, their discovery makes possible timely isolation of contact individuals thus preventing further disease spread.

Oral Manifestations of HIV Infection

Severe immune suppression that follows progressive ***HIV infection*** results in development of ***acquired immune deficiency syndrome***, or ***AIDS***. Similar with other body compartments, HIV dampens immune response within oral cavity. This creates conditions for local oral manifestations of opportunistic infections and tumors.

Before the clinical implementation of highly active antiretroviral therapy (or HAART) in 1996, oral lesions were registered in 50% of individuals infected with HIV and in about 80% of persons with AIDS. Since that time the course of HIV infection has become more benign but the oral lesions remain to be common in this patients.

In 1992 the EC-Clearinghouse on oral problems related to HIV infection and WHO Collaborating Centre on Oral manifestations of the immunodeficiency virus worked out the classification of oral manifestations of HIV infection. Later, in 2002, an international workshop confirmed the validity of this classification and recommended it for practical use.

Classification data summarizing oral lesions associated with HIV/AIDS are present in Table 11.

The most common oral lesions in HIV/AIDS are the results of ***candidal infection***. They occur at least in 40-60% of AIDS patients. The main causative agent is *Candida albicans*; it often associates with *C. tropicalis*, *C. parapsilosis* and others. *Erythematous* and *hyperplastic* candidiasis, *chronic pseudomembranous* disease, and *angular stomatitis* are typical in AIDS.

Viral infections in HIV/AIDS rapidly progress in condition of severe cellular immune deficiency. The viruses from ***Herpesviridae*** family play pivotal role in genesis of viral complications in AIDS.

Among them are herpes simplex and herpes zoster viruses, herpesvirus type 4 or Epstein-Barr virus, cytomegalovirus (herpesvirus type 5), and herpesvirus type 8 or Kaposi's sarcoma-associated herpesvirus.

Active reproduction of *Epstein-Barr virus* in epithelial cells of tongue exerts its specific lesion, known as ***hairy leukoplakia***. It follows about 50% of HIV infection cases.

Hairy leukoplakia represents white wrinkled lesions from lateral sides of tongue that are not painful. In most of cases it doesn’t need specific treatment. Re-emergence of hairy leukoplakia in the same patient may indicate the failure of HIV specific therapy.

Table 11
Oral lesions, associated with HIV/AIDS

Group 1. Lesions strongly associated with HIV infection	***Group 2. Lesions less commonly associated with HIV infection***	***Group 3. Lesions seen in HIV infection***
	Bacterial infections:	***Bacterial infections:***
Candidiasis	Mycobacterium avium-intracellularae Mycobacterium tuberculosis	Actinomyces israelii Escherichia coli
Hairy leukoplakia	Necrotizing (ulcerative) stomatitis	Klebsiella pneumonia Cat-scratch disease
Kaposi's sarcoma	Salivary gland disease	Bacillary angiomatosis
Non-Hodgkin's lymphoma	Dry mouth due to decreased salivary flow	**Fungal infection other than candidiasis:**
Periodontal diseases: linear gingival erythema, necrotizing ulcerative gingivitis, necrotizing ulcerative periodontitis	Unilateral/bilateral swelling of salivary glands Non-specific ulcerations	Cryptococcus neoformans Geotrichum candidum Histoplasma capsulatum Mucoraceae fungi Aspergilus flavus
	Viral infections:	
	Herpes simplex virus Human papillomavirus Condyloma acuminatum Varicella-zoster virus	***Neurological disturbances:*** Facial palsy Trigeminal neuralgia

Herpesvirus type 8 causes angioproliferative tumor ***Kaposi's sarcoma***. It affects one third of patients with AIDS. About 40% of them demonstrate oral lesions. This vascular tumor renders multiple red-violet macules, papules or nodules mainly upon hard palate.

Repetitive herpes zoster infections are also common in AIDS patients.

Deep immune suppression in patients with AIDS-related complex and AIDS activates numerous opportunistic pathogens resulting in severe mixed bacterial, fungal and viral infections.

Among the most common are tenacious periodontal disorders of polymicrobial origin.

Usually periodontal damage starts from ***linear gingival erythema*** or *HIV-associated gingivitis*. It may progress further into ***necrotizing ulcerative gingivitis*** and ***necrotizing ulcerative periodontitis***.

Linear gingival erythema looks like narrow red band surrounding the marginal gum. If not controlled, it stimulates progression of *necrotizing*

ulcerative gingivitis with deep damage of papillary gingival area and *necrotizing ulcerative periodontitis* with dental attachment loss and destruction of alveolar bone.

Periodontal pathology in AIDS arises from the joint action of typical oral pathogens, such as gram-negative obligate anaerobes *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Actinomyces naeslundii*, and others. *Aggregatibacter actinomycetemcomitans*, candidae fungi, and viruses are also the active participants of AIDS-associated periodontal diseases.

Учебное издание

Генералов Игорь Иванович

МЕДИЦИНСКАЯ МИКРОБИОЛОГИЯ, ВИРУСОЛОГИЯ И ИММУНОЛОГИЯ

для студентов лечебного и стоматологического факультетов высших медицинских учебных заведений

Пособие

Редактор И.И. Генералов
Технический редактор И.А. Борисов
Компьютерная верстка А.К. Гайлит
Корректор Н.В. Железняк

Подписано в печать
Формат бумаги 64x84 1/16 Бумага типографская №2.
Гарнитура ТАЙМС. Усл. печ. листов . Уч.-изд. л
Тираж экз. Заказ № .

Издатель и полиграфическое исполнение:
УО «Витебский государственный медицинский университет»
ЛП № 02330/453 от 30.12.2013 г.
Пр. Фрунзе, 27, 210602, г. Витебск