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DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

MICROBIOLOGY – PRINCIPAL AND INTERPRETATION OF LABORATORY EXAMINATION PART 1

Textbook for supplementary study in microbiology, clinical microbiology and laboratory diagnosis of microbial ethiology of diseases

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This textbook is dedicated to English speaking students and teachers interested in laboratory microbiology and serology. Our intention was to spread the knowledge for better understanding the role of these laboratory branches and their role in the diagnostical process. We are thankfull for the support and help from cited authors, collegues, students and our families. All materials are presented to be used for study purposes.

Contents

INTRODUCTION

Microbiology is a broad field concerned with the study of microorganisms. It is divided into a number of subdisciplines reflecting the variety of microorganisms that it studies. These subdisciplines include bacteriology, virology, mycology and parasitology. It also overlaps with disciplines such as anti-infection immunology, which studies how macroorganisms respond to infection by a microorganism, and serology, which is concerned with the diagnostic significance of specific molecules in serum and their detection.

Medical microbiology – is a discipline that incorporates *microbiology* into the study of medicine, focussing on the relationship between microorganisms and human beings, especially microorganisms' pathogenic potential. It is usually studied as a pre-clinical subject, as at the Jessenius Faculty of Medicine in Comenius University. It is very closely linked with clinical fields in practical medicine because of its relevance for determining the relationship of isolated and identified microorganisms to human diseases in the stage of pathogenesis, acute illness, convalescence or chronic illness. It studies the interaction between the defensive mechanisms of the macroorganism and the pathogenicity mechanisms of an infectious agent. For conditionally pathogenic microorganisms (physiological flora, nasal strains), it studies the changes in the interaction, which means the risks resulting from the failure of non-specific defence mechanisms or the conditions for the transmission of an infectious agent to susceptible individuals.

All aspects of medical microbiology are linked to diagnostics. **Microbiological examination** is the examination of biological samples taken by clinical practitioners to identify infectious agents based on a set of clinical signs indicative of a possible infectious origin (the microbial aetiology of a disease). In microbiological testing, laboratory diagnostics is used to solve the problems of differential diagnosis arising from the non-characteristic symptomatology of most infectious diseases and diseases with a microbial aetiology. The clinical signs of infections are generally non-specific and can be caused by several microorganisms often belonging to different taxonomic groups. Besides identifying the aetiological factor, or determining its susceptibility to antimicrobial drugs, the most important part of the work of doctors in **clinical microbiology** is to cooperate with other clinical practitioners. Cooperation is necessary in the interpretation of laboratory test findings to determine the role of an isolated and identified microorganism in the pathogenesis of the given disease process. It includes participation in joint decisions on

aetiological therapy for a patient whose biological material has undergone microbiological examination. Clinical microbiologists use their experience and knowledge of microorganisms and antimicrobial medicinal substances to estimate the relationship between the result of the *in vitro* examination and the possible action of the microorganism *in vivo*. Repeated examinations verify the success of medical procedures, record indications of possible failure of anti-infective therapy, and any changes that may be needed.

In certain cases, information on the results of identification and the characteristics of an infectious agent requires further processing (reporting, confirmation by a reference laboratory, preservation for further investigation) and are the basis for further medical, scientific or forensic activities by other practitioners. These cases include situations where there is a threat to public health or where it is necessary to protect against the abuse of biological resources for war or terrorism. In the present time, microbiological laboratories and microbiologists are also involved in searching for and identifying emerging and re-emerging pathogens in the changing spectrum of causal agents of infectious diseases. New microorganisms are continuously emerging as aetiological agents, conditionally pathogenic microorganisms are being triggered, the causes of previously controlled infections re-emerge thanks to the failure or incomplete application of the public health authorities' protective measures, and there is the risk of microorganisms may be misused in warfare or bioterrorism.

1 LABORATORY AND CLINICAL MICROBIOLOGY

Microbiology is a broad discipline that can be defined as the study of phenomena and interactions on the level of the microworld of living systems (living organisms that are invisible to the naked eye). It studies microorganisms' structure, metabolism, biochemical processes and the mechanisms of their pathogenicity and virulence, their effects on target structures and the environment, their susceptibility to antibiotics (ATB), and the mechanisms by which microorganisms acquire resistance to ATB.

Medical microbiology a discipline that applies scientific knowledge of microbiology to the field of medicine. Medical microbiology studies medically significant microorganisms, their structure, metabolism, biochemical processes, the pathogenesis of the diseases that they cause, the mechanisms of microbial causal agents' pathogenicity and virulence, their susceptibility to antibiotics (ATB), the mechanisms by which they acquire resistance to ATB, and the mechanisms by which human immune systems defend themselves against microorganisms. It is also concerned with the detection of such microorganisms by laboratories. The basic groups of medically important microorganisms are:

- bacteria,
- fungi,
- protozoa.

Microbiology also studies non-living structures that are able to cause infectious disease in humans:

- viruses,
- prions.

Microbiology, especially its parasitology branch, uses microbiological diagnostic methods to investigate and study the anatomy, vital signs and significance of certain macroorganisms in causing infectious diseases:

- worms

and in the spread of infections:

- arthropods.

Clinical microbiology uses knowledge from microbiology and medical microbiology in:

- diagnostics (identification of microorganisms),

• therapy (determining their susceptibility to anti-microbials),

• the prevention of diseases with microbial aetiology

by assessing the interaction of a microorganism's pathogenicity mechanisms and pathogenic potential, and the organism's defence response against them.

In the healthcare system of the Slovak Republic, clinical microbiology is performed in autonomous laboratories belonging to the system of common examination and treatment units (in Slovak: *spoločné vyšetrovacie a liečebné zložky* – SVLZ) The basic organisational unit in clinical microbiology is a **clinical microbiology department** (in Slovak: *oddelenie klinickej mikrobiológie* – OKM). Depending on the scope and types of activities that a department performs, it may be divided into a number of sections or laboratories. In exceptional cases, a specialised clinical microbiology laboratory may form part of another organisational unit such as a public health authority (in Slovak: *úrad verejného zdravotníctva* – ÚVZ).

Possible specialisations for laboratories within a clinical microbiology department include:

- bacteriology,
- cultivation of mycobacteria,
- virology,
- anti-infection immunology,
- serology,
- mycology,
- parasitology:
  - protozoology,
  - helminthology,
  - entomology.

Other important functions include the preparation of culture media and solutions, and quality control. Reference laboratories conduct additional diagnostic activities, oversee quality control for laboratory work and perform activities requiring higher-level expertise in clinical microbiology.

Public health includes a separate discipline concerned with the objective identification of environmental factors – **environmental microbiology**. It is concerned with evidence of the incidence of microorganisms in the environment, in water, in food, in items of daily use and non-living structures where they could influence humans, their health or their activities, for example

sterility checks for operating instruments. Activities in this area are under the competence of public health authorities.

The main funcion of clinical microbiology is to provide comprehensive microbiological diagnostics to determine the aetiology of microbial diseases and causal anti-infective or immunomodulatory therapy for patients. This includes the diagnosis of diseases caused by pathogenic bacteria, viruses, microscopic fungi, parasites as well as opportunistic pathogenic microorganisms that may play a role in disease pathogenesis in patients with immune disorders. It demonstrates the presence of physiological microbial flora on human skin and mucous membranes and helps to objectively determine and assess patients' health condition. Its diagnostic activities make use of the possibility to establish direct proof of an aetiological agent in biological materials. For this purpose, it makes use of microscopy, evidence of antigens, nucleic acids, cultivation, isolation on media and procedures for identifying the originator of a disease. Standard examination for bacterial and fungal diseases includes determining susceptibility to antimicrobial drugs. Methods for the indirect diagnosis of microbial disease include serological methods for detecting antibodies against the antigens of microorganisms and identifying indicators of an inflammatory response and immune status. All these activities are defined in the conception of the field as part of the system of medical-preventative activities directed by the Ministry of Health of the Slovak Republic. At present, microbiology in the Slovak Republic is classified under the subject code 4.2.7 under sub-group 4.2 life sciences within the natural sciences. It is not classified under group 7 (health) with other medical disciplines (http://www.minedu.sk/).

2 MICROBIOLOGY AS A SCIENCE

Microbiology (from the Greek μῑκρος, *mīkros*, "small"; βίος, *bios*, "life" and -λογία, *-logia*, science) is the scientific discipline that studies microorganisms, which are microscopic single-cell organisms or cell aggregates. These include eukaryotic organisms (such as fungi and protists) and prokaryotic organisms. Viruses and prions are not classified as living organisms. They are nevertheless studied as part of microbiology. Microbiology also includes the study of the defence mechanisms of susceptible individuals in general and against the potential pathogenic effects of microorganisms (immunology, anti-infection immunology).

Microbiology is a scientific discipline in active and progressive development. It is estimated that just 1% of all microorganisms on the planet have been studied (Amann, 1995). Although the first microorganisms were observed around 300 years ago, microbiology became established as a science later than the older biological disciplines (botany, zoology) and it remains a relatively young science.

Several subdisciplines have been established within microbiology. There are significant overlaps between microbiology and related fields such as biology and epidemiology as well as other scientific disciplines outside medical science.

The scientific disciplines of microbiology can be classified according to several schemes: (http://en.wikipedia.org/)

Taxonomical – reflecting the taxonomic differences between microorganisms:

- bacteriology: the study of bacteria
- mycology: the study of fungi
- protozoology: the study of protozoa
- phycology (algology): the study of algae
- parasitology: the study of parasites

Integrated – the application of other biological disciplines to microorganisms:

- cytology of microorganisms: the study of the microscopic and sub-microscopic details of microorganisms and their cell structures,
- physiology of microorganisms: the study of the biochemical functions of cells including the study of the growth, metabolism and function of cellular structures,

- genetics of microorganisms: study of the organisation and regulation of genes in microorganisms in relation to their cellular functions; this discipline is very closely related to molecular biology.
- evolutionary microbiology: the study of the evolution of microorganisms; this discipline can be subdivided into:
    - the taxonomy of microorganisms: the study of the nomenclature and classification of microorganisms,
    - the systematics of microorganisms: the study of the differences and genetic relatedness of microorganisms.

Others:

- nanomicrobiology: the study of microorganisms on the nano level,
- exomicrobiology (or astro microbiology): the study of microorganisms in outer space.

Applied:

- medical microbiology: the study of pathogenic and medically significant microorganisms, their function in the pathogenesis of human diseases; it includes the study of the role of microorganisms in onset of the disease, the status of microorganisms in the spreading of infections and it has close links to the study of pathology, immunology and epidemiology,
- pharmaceutical microbiology: the study of microorganisms relevant to the production of antibiotics, enzymes, vitamins, vaccines and other pharmaceutical products, and which cause pharmaceutical contamination,
- industrial microbiology: the study of microorganisms for industrial use, for example in industrial fermentation, in water treatment and in brewing; this discipline is closely related to biotechnology,
- microbial biotechnology: the study of the manipulation of microorganisms on the genetic and molecular level to create usable products,
- food microbiology: the study of microorganisms' role in food spoilage and food-borne diseases, and the use of microorganisms in food production, for example through fermentation,
- agricultural microbiology: the study of microorganisms of significance for agriculture; this discipline can be further subdivided into:

    - microbiology of plants: study of the interaction of microorganisms, plant pathogens and plants,
    - microbiology of soil: the study of microorganisms present in soil,
- veterinary microbiology: the study of microorganisms of significance for veterinary medicine, their function in veterinary medicine and the taxonomy of animals,
- environmental microbiology: the study of the function and diversity of microorganisms in their environment and their relations; it includes the characterisation of the key habitats of microorganisms such as the rhizosphere and the phyllosphere, soil and groundwater ecosystems, the open seas and extreme environments,
- microbial ecology: the study of microorganisms with reference to the human environment,
- water microbiology: the study of microorganisms found in water,
- air microbiology: the study of airborne microorganisms.

Despite the undeniable concerns raised by the pathogenic effects of medically significant microorganisms on humans, some microorganisms have uses in processes where they have a positive effect on human life and provide benefits. Examples include industrial fermentation, which is a process in the production of alcohol, vinegar, dairy products, cheese and beer, the production of antibiotics, the use of microorganisms as a substrate for the cloning of more complex organisms, or as enzyme transfer vectors or the like. Scientists have used their knowledge of microorganisms for the biotechnological production of the enzyme Taq polymerase, which is used in molecular biological detection systems. All the scientific disciplines studying microorganisms and their properties aim to develop practical applications for them (Madigan, 2006).

There are several microorganisms that can serve as examples. *Corynebacterium glutamicum* is used in the industrial manufacture of amino acids. It is a very important bacterial species which is responsible for producing more than two million tonnes of amino acids per year (Burkovski, 2008).

microorganisms produce several polymers (polysaccharides, polyesters, polyamides). They are used in the biotechnological production of biopolymers with defined properties suitable for the most demanding medical uses (tissue and drug production, the biosynthesis of hyaluronic acid, oligosaccharides, polysaccharides; Rehm, 2008).

Another useful property of certain microorganisms is their ability to degrade products (biodegradation of waste, polluted soil, sediments, water). The most effective method of microbial degradation is to use a mixture of species and strains, each of which specialises in breaking down one or more contaminants (Diaz, 2008).

A well-known and widely used way in which microorganisms contribute to health is in the form of probiotics (microorganisms with a potentially beneficial effect on the digestive system) and prebiotics (components supporting the reproduction and action of probiotics). The production of such microorganisms is part of industrial or pharmaceutical microbiology (Tannock, 2005).

Recent studies have identified new therapeutic uses of microorganisms, for example in the treatment of cancer. Various non-pathogenic clostridia infiltrate solid tumours and replicate in them. These microbial vectors can be safely administered and their potential to transport therapeutic proteins to the required site has been demonstrated in several preclinical and experimental models (Mangesha, 2009).

3 MICROBIOLOGY AS A DIAGNOSTIC TOOL

Infectious diseases and their diagnosis, treatment and prevention consume a large part of a state's healthcare budget. While treatment is the largest cost for developing countries, the heaviest financial burden for developed countries is in the diagnosis of infectious diseases. Prudence should be exercised when requesting microbiological tests. It is not a good idea to ask for every test that a laboratory offers. It is useful to limit a test to what will assist the diagnosis. Although laboratories can reduce subjectivism and the risk of individual error in diagnoses by applying standard methods, algorithms and calibrated instruments that replace the manual and repetitive (not thoughtless) work of laboratory personnel, a medically trained microbiologist must make an individual assessment of the methods to be used, their timing and especially of the interpretation of the results for every biological sample (Vandepitte, 2003).

To accomplish this task, microbiological laboratories are established to provide diagnostic services to hospitals and outpatient doctors, and perform additional activities. The complexity of laboratories' work and their specialisation should increase from local to regional and central institutions. There is currently very little risk that microbiological laboratories, especially those that provide direct diagnostic services, will be replaced by automatic testing units of the sort that can be found in some of the big laboratories at the centre of the SVLZ lab network. There is, however, a risk of a "results overload", which results in the wastage of resources. Testing can produce so much data that it becomes an uncontrolled and impenetrable forest in which it can be hard for clinicians to find their way. A key factor is the time between a sample being taken and the delivery of results to the clinical practitioner.

Infectious diseases are the most common cause of death and their diagnosis and treatment is a major challenge for healthcare in all countries. The WHO laid down the first technical and professional standards for diagnostic and research laboratories in 1960 (WHO, 1977). The WHO also made the first attempt to introduce standardised quality control in 1981 with the IEQASM – International External Quality Assessment Scheme for Microbiology (WHO, 2003).

An infectious aetiology can be diagnosed using two basic methods: direct identification of a microorganism and indirect evidence of infectious aetiology based on specific markers of infection in serum and tissues.

Direct diagnosis of an aetiological agent means the visualisation of a microorganism, or parts or physiological properties of a microorganism sufficient for its identification. A not very extensive range of diagnostic instruments are usually sufficient for the performance of a complete microbiological examination that makes a significant contribution to the individual patient's treatment and care by establishing an aetiological diagnosis. The ability of a microbiological laboratory to perform this function is limited by the quality of the sample taken for the patient, its transportation and the technical conditions for detecting the microorganism in the sample. Direct diagnosis methods are usually dependent on a microorganism's ability to grow and therefore transport and cultivation conditions must ensure the viability of the pathogen. Another reason for isolating a viable pathogen, besides identification, is to study the antimicrobial activity of selected preparations that could be used in treatment. The basic methods for the direct diagnosis of the cause of an infection include microscopy, cultivation, molecular diagnostics and serological diagnostics of antigens and microorganisms.

The use of microscopy in microbiology has two fundamental objectives: preliminary detection of microorganisms and preliminary or definitive identification of the microorganism. Microscopic examination of a clinical specimen is used primarily to detect bacterial cells, fungi and their parts, parasites (eggs, larvae, adult forms) and viral inclusions present in infected cells. Typical morphological characteristics can provide a preliminary identification of most bacteria and a definitive identification of many fungi and parasites. Microscopy can also be used for the rapid identification of microorganisms by using an antibody bearing a fluorescent colour or another marker. Medical microbiology uses five microscopic methods adapted to different microbial characteristics and different objectives of examination: bright-field microscopy (unstained or stained), dark-field microscopy, phase contrast microscopy, fluorescence microscopy and electron microscopy (Gest, 2005).

Cultivation exploits the ability of microorganisms (bacteria and fungi) to grow on artificial media, which remains an important part of the toolkit of every clinical microbiology laboratory even in the era of rapid methods and molecular techniques. For many diseases, the cultivation of a microorganism from the site of infection is the definitive method for identifying the cause of

the disease. The success of cultivation methods depends on the biological properties of the organism, the site of infection, the patient's immunity and the quality of the culture medium. Biological samples may need to be inoculated into multiple media and further media will be needed for identification. It is therefore particularly important to select culture media and procedures with care when a sample is small or there are problems with sampling or its repeatability. The microbiologist and the clinician must cooperate to take the most useful sample at the most appropriate time. In many cases, this increases the probability of isolating and identifying the aetiological agent.

Cultivation methods reproduce the microorganisms for further tests leading to the identification of the strain. These identification methods are phenotypic and look for evidence of a microorganism's biochemical properties – usually its metabolic enzymes or pathogenicity mechanisms. Colonies of microorganisms are also the substrate for a modern diagnostic procedure based on protein identification – proteomics (the MALDI TOFF method),

As in human biology, microbiology can work with the DNA, RNA or proteins of an infectious agent in a clinical sample to give it a precise identification using molecular methods and proteomic methods (Amann, 1995). In many cases, these methods are able to identify an aetiological agent that could not be isolated and identified by immunological methods. The advantages of molecular techniques are their sensitivity, specificity and safety. The advantage for safety is that these methods do not require the isolation and reproduction of the infectious agent. They can be applied to inactivated or fixed specimens. The agent can be identified even if it does not reproduce in tissues. The techniques are also able to distinguish between related strains based on genotypes. Another important use of molecular methods is in finding resistant strains (mutants), which can differ in a single nucleotide. The identification of an aetiological agent can be speeded up further by setting up a multiplex system that offers primers to test biological samples for the commonest pathogens. Examples of such systems include FilmArray, Idahoo, Iridica. They offer simpler diagnoses, several hours' less work, more accurate identification, including species-level identification, even for unusual aetiological agents. Despite high initial costs and often also high operating costs, they provide a large economic benefit by greatly shortening the diagnostic and therapeutic process. It allows the use of highly targeted

antimicrobial therapies because it has the potential to identify resistance genes as well. Knowing the pathogen reduces the need for empirical therapy and wide-spectrum antibiotic therapy, which is one of the reasons, although only one of the reasons, for the uncontrolled growth of antibiotic resistance.

A laboratory tests microorganisms' susceptibility to antibiotics. It tests only the microorganisms that are able to cause disease and tests them only against therapeutically useful antimicrobial medications. Testing all isolated microorganisms or unsuitable medications could lead to the incorrect interpretation of the results with potentially undesirable consequences. Not only could the patient receive unsuitable treatment, but the actual pathogen could escape detection. A finding of susceptibility in vitro is only a laboratory image for estimating the situation in the body. The interaction between the patient and the microorganism is affected by the microorganism's virulence and quantity, the site of infection and the patient's ability to respond to the infection, and all these factors must be considered when planning treatment. Microbiological examinations are also the basis for the regular monitoring of the development of antibiotic resistance. They help to decide which antibiotics should be considered in empirical therapy. They are an information source for tracking resistance trends, estimating regional differences in resistance and analysing their causes.

The growth of microorganisms' resistance to antibiotics is becoming an important issue and some agents are already unaffected by any currently available antibiotics. Strains are emerging for which no known antibiotic is available or just one from the whole range (for example certain MRSA, *Pseudomonas aeruginosa*). This problem is partly due to irrational interventions in the ecology of humans and microorganisms such as the excessive and incorrect prescription of antibiotics, the use of antibiotics in agriculture, imprudent antibiotic policies in hospitals and in the community and the irrational use of wide-spectrum antibiotics.

The empirical use of antibiotics must be governed by the fundamental rules of rational pharmacotherapy. Antibiotics (in the strict sense, meaning antibacterial antibiotics) are used for infections caused by bacteria. The cause can be identified quickly based on a clinical picture, experience and auxiliary examinations such as CRP measurement, where elevated values are evidence against a viral infection. Considering that most respiratory tract infections are caused by viruses, but they are still most frequently treated with antibiotics, better use and interpretation of

CRP testing would help to reduce the amount of unnecessary antibiotic use. Further reductions in antibiotic consumption can be achieved using data on the most frequent causes of infections and natural resistance, and information on resistance levels in the area. Empirical antibiotic use must be supported by regular testing and a current assessment of the aetiological agent's susceptibility to selected antibiotics in a given region. The method used to select target strains depends on the range of samples tested. It is necessary to apply the rules of random selection such as testing susceptibility just one week in a month or a certain day in the week. Such a procedure is in accordance with the patient's interests and the rational use of antibiotics (Hoza, 2002; Hupková, 2010).

Antibiotics policy provides the guidelines for the safe and effective use of antibiotics. Microbiological examination is an important tool for its development. The rules on antibiotic use should ensure the required efficacy and safety of antimicrobial therapy, limit the emergence and spread of resistant microorganisms and to reduce the consumption of antibiotics. Antibiotics are preparations whose purpose is to eliminate a pathogen and its effects and allow the macroorganism to deal with an infection using its own immune system. The effective targeting of antibiotic therapy requires the proven identification of the infection agent and determination of its susceptibility to antibiotics. Empirical use is based on current, relevant epidemiological data on the likely aetiology of the infection and the resistance of possible agents. This cannot be achieved without microbiological examinations correctly performed according to set procedures (Steinman, 2003). Some analyses of antibiotic consumption and the occurrence of resistance in studied areas have suggested that the intensity of antibiotic exposure in the community is correlated with an increase in resistance. On the other hand, many works have found no correlation between exposure levels and ATB resistance (Doczelová, 2004; Kafetzis, 2004; Winkelstein, 2001). Specific resistance phenomena may be triggered by selection pressure independent of the structure of consumption and the quantitative representation of individual ATB groups. They can be detected through the regular analysis of large (statistically significant) numbers of examinations of the susceptibility of samples in a microbiological laboratory.

Serological methods are mainly used in indirect diagnostics and provide evidence of an antibody response to infection or exposure to infectious agents. They can, however, also be used to identify viruses or other agents that are difficult to isolate or that cannot be grown in laboratory

conditions. They are therefore used to detect, identify and quantify microbial antigens in a clinical specimen. They are also used to narrow the identification of antigens – serotyping – by determining the antigen structure of microorganisms. High-sensitivity serological tests such as latex fixation tests are used for the rapid diagnosis of free antigens in biological material (e.g. CSM, urine, blood). The significance of serological responses within laboratory work depends on the specificity of antigen-antibody interactions and the sensitivity of different immunological techniques.

Indirect diagnosis of an aetiological agent is based on the identification of specific antibodies. Serology is the scientific discipline that studies the components of blood serum and other body fluids. In practice the term is used to refer to the diagnostic identification of antibodies in serum. Antibodies are typically produced in response to an infection to combat a specific microorganism, a specific extraneous protein or, in an autoimmune disease, the organism's own structures. Serology can be used in an indirect diagnosis procedure to determine the history of the patient's exposure to antigens, to plot the course of infection or to determine the type of infection as a primary infection or reinfection, or as an acute or chronic infection. The type of antibodies and their concentration provide serological data on the infection and its stage. The concentrations of antibodies are usually quantified as the number of international units per litre (IU/l). The relative concentration of antibodies is referred to as the titer. The titer is the inverse of the greatest dilution of a patient's serum sample that gives a positive antigen-antibody reaction. Serological reactions are used to determine the stage of infection based on identification of the antibodies against the various types of antigens produced by the causal agent. The first antibodies present during an infection are directed against antigens with the greatest exposure to the immune system (surface antigens of the agent, antigens present on the surface of an infected cell). Later in the infection, when cells are damaged and lysed, antibodies are directed at proteins and enzymes present in the intercellular medium. Serological tests also assist the diagnosis of some immune disorders, especially those associated with inadequate antibody production, such as X-linked agammaglobulinemia and AIDS In this case, tests for the presence of antibodies, including specific tests, are negative. Similarly, an IgA test used in the diagnosis of acute disease will be negative in the event of the most common immunodeficiency, IgA immunodeficiency.

Serological tests are not limited to blood serum but are also used to detect antibodies and other markers in saliva, transudate, cerebrospinal fluid, ejaculate or fluid obtained by paracentesis. Finds of antibodies and measurement of relative concentrations in serum and other bodily fluids (most often in cerebrospinal fluid) can be used to infer the presence of local infections. An example is evidence of intrathecal antibody production against CNS central nervous system infections.

The aggregate results of serological examinations – immunological surveys – are used prepare information on the prevalence of diseases in the population or to determine the specific immunity of populations or population groups. These surveys are conducted by the random, anonymous examination of samples taken for other diagnostic tests, which are selected according to well-defined criteria or by collecting anonymous samples from volunteers according to established criteria.

Estimating the risk of occurrence of communicable diseases requires the monitoring not only of individual immunity but also the monitoring of collective immunity in order to protect the non-immune populations and populations that cannot be vaccinated. Such monitoring is part of the epidemiological surveillance system for communicable diseases. Serological tests can give an objective picture of the population's specific immunity for a chosen disease. They are the basis of immunological surveys that enable public health bodies to identify an immunological hole or a trend in the development of the specific immunity of a certain group or the overall population. Regular immunological surveys allow immunisation schemes to be modified for vulnerable groups or the entire population. They can confirm the correct and effective timing of vaccination schedules. The basis of a good immunological survey is to collect samples in accordance with the requirements for statistical data sets, to perform adequately sensitive and valid tests and to evaluate and interpret the results correctly. It is essential to standardise working procedures at all levels of the organisation participating in immunological surveys, but especially in the microbiology laboratory (file selection, sample coding, sampling, processing, sample storage, transport, sample handling, examination, recording and archiving of results, statistical processing). Immunological surveys are conducted for the following purposes:

– one-time verification of an antibody's presence in an immunologically unknown population,

– identifying antibody dynamics over a certain period in different age groups (samples taken at one collection date),

– creating a longitudinal survey by prospectively monitoring the antibody concentrations in one stable group that is followed for a long period (cohort).

When selecting a group, statistical requirements for a data set must be respected. Blood samples must always be taken under the same conditions. Each blood sample generally undergoes testing for multiple parameters and therefore at least 5 ml of blood is taken so that each test has at least 0.5 ml of serum. For each sample a set of documents must be prepared in advance: a questionnaire, the identification label for the sampling kit, an identification label with a code for each test tube for each test separately. The individual questionnaire includes general information, personal data and epidemiological data. A common epidemiological description is prepared for each group – the selection criteria based on which the group was created.

The special characteristics of the examination of serum samples for an immunological survey include:

– the selection of a suitable methodology for detecting a certain type of antibodies (selection of the antibody class, methods for the antibody type – CFR (complement fixation test) or VNT (virus neutralisation test) for poliomyelitis, IgG antibodies against diphtheria or antitoxic antibodies against the toxin *Corynebacterium diphtheriae*),

– standard conditions all examination cycles,

– standard conditions during the whole examination period,

– solutions for changes in conditions during longitudinal monitoring (concentration conversion, index generation),

– quality control (internal, external, procedure blanks, sample anonymity),

– determination of the antibody concentration for a positive test,

– determination of the antibody concentration expected to provide protection against the onset of the disease.

Interpretation of laboratory results is an activity that adds value to every microbiological examination. The expected outcome of a microbiological examination is affected by multiple

objective and subjective factors and it is only a part of the auxiliary information that a clinical practitioner can draw on in making a diagnosis and selecting a suitable therapeutic procedure. The factors that directly affect the quality of a microbiological result include the sampling procedure for the material, its timing and technique, the transport of the biological sample, the selection of the examination method used and the quality and level of all laboratory activities and the verification of the result. The spectrum of examinations performed by a clinical microbiology department is determined by the requirements of the clinical practitioners. The role the microbiological laboratories is to identify the causal agent of a disease. To accomplish this, it uses a range of laboratory procedures (microscopy, cultivation, fluorescence, enzyme analysis, radionuclide analysis, serological methods, NK (natural killer) cell detection etc.) Most of them have practical uses outside microbiology. Genetic methods for identifying microorganisms are no more the sole property of microbiologists than determining the biochemical properties of microorganisms is solely in the domain of a biochemistry laboratory (Riordan, 2002). The examination is just one of a series of actions whose quality determines whether or not the right result is found. The technical implementation of an examination is largely determined by the quality of the diagnostic set and the extent to which the laboratory that applies GLP (good laboratory practice) principles. The interpretation of results concerning the cause of disease must be made by the diagnosing physician and a specialist in the relevant area, but they can consult with the microbiologist (Thomson, 2004).

Prenatal screening to detect the presence of an intrauterine foetal infection or to eliminate the suspicion of the occurrence of a vaccine-preventable disease in a vaccinated person requires a responsible approach, an accurate algorithm and correct interpretation of the data obtained by the laboratory. In our population, it is obligatory to be vaccinated against certain viral diseases (rubella, measles, parotitis). There are very few women of fertile age who were not vaccinated against rubella and also very few people who were not vaccinated against the other mentioned infections or who did not overcome the diseases in childhood. Nevertheless, it is impossible to exclude the possibility that there are individuals in our population who do not have protective antibodies (residents of countries that do no vaccinate, individuals who were not vaccinated, immunodeficient individuals, non-responders). Such people are at risk of acute infection and potential susceptible individuals, even though the occurrence of disease is unlikely from an

epidemiological point of view. It is possible for an aetiological agent to be introduced from a country where collective immunity is insufficient to prevent its spread (85%). In such an epidemiological situation where there is suspicion of the occurrence of a vaccine-preventable disease or where prenatal screening raises the possibility of intrauterine rubella virus infection, the patient is tested for the presence of IgG antibodies (testing for protective immunity) and if this test gives a negative result they are tested for the presence of IgM antibodies (screening for acute infection). Great care must be taken in the interpretation of a positive result for IgM antibodies. Practitioners must also consider the clinical picture, the epidemiological history and the dynamics of the antibody concentration. The interpretation of serological tests must consider the level of antibody concentration expressed in international units or at least indexes of semi-quantitative evaluation. Every off-the-shelf or laboratory-made test has a limit (cut-off) for the concentration of the antibodies it detects or the achievement of extinction, which constitutes the boundary between a positive and negative result. The boundary value of an anti-body determining test is generally determined based on the values most frequently obtained when investigating large sets. Careful consideration must therefore be exercised when values are close to this boundary. The range 10% above or below the cut-off value is a grey area indicating an unclear result. Mentioning the grey area in a laboratory result is not recommended, because it increases the level of uncertainty in interpretation. Serological tests have certain objective and subjective limitations, which must be borne in mind. A false positive result can be produced by cross-reactive antibodies produced after exposure to antigen-like agents. In some diseases, and especially in pregnant women, there can be polyclonal activation of cells (non-specific cell activation and polyclonal antibody production) and this can cause a situation in which tests for multiple antigens produce several positive results (IgM classes in low positive concentrations). The cause may be the existence of a rheumatoid factor, IgM-type antibodies against an immunoglobulin Fc fragment. There are ways to remove these non-specific antibodies in the laboratory preparation process (Doan, 2008).

An example of the importance of test result interpretation is testing for the presence of HPV (human papilloma virus) infection. Detecting this viral infection is important because of the availability of a vaccine against some types of high-risk HPV strains. Finds of such strains in a patient’s biological sample must be interpreted in combination with the local clinical finding,

which is the ultimate determining factor for further treatment or a prophylactic procedure (Hausen, 2002). The presence of low-risk strains does not imply the presence of non-risk strains. Vaccination against HPV carries the risk of “failing” when incorrectly informed. Vaccination does not mean that preventive examinations can be scaled back because the vaccine protects against only a few of the most common strains representing the highest risk and cannot prevent infection by strains not contained in the vaccine. It provides no protection against a malignant disease already infecting a person. It can only prevent future infection (Sankaranarayanan, 2005).

4 MICROBIOLOGY AS AN INSTRUMENT OF SURVEILLANCE

Many communicable diseases are successfully controlled or even eliminated through preventive measures. Nevertheless, factors can emerge that indicate the risk of microbial epidemics. These factors include changes in microorganisms' ecology, the emergence of new pathogens, the re-emergence of pathogens previously controlled by procedures for preventing the onset and spread of infections, the involvement of conditional pathogens in the disease processes of people living with significant immune disorders (Weiberg, 2005).

The procedures used in medical microbiology include many epidemiological methods, notably epidemiological surveillance. One of the fundamental requirements of surveillance is the precise identification of the aetiological agent of the infectious disease being monitored. Epidemiological approaches also use medical microbiology in the surveillance and control of antibiotic resistance and provide evidence for the surveillance and control of hospital-acquired infections.

An epidemiological surveillance programme is a system of work designed to provide information for further activities. It can function on multiple levels and address a wide range of issues (surveillance of the ATB resistance development of selected pathogens in a region, surveillance of blood-borne diseases in dental practice, nationwide surveillance of Hib infections, European surveillance of *Clostridium difficile* infections – ECDIS). A surveillance strategy can have different levels of detail. It can be a "full" or "light" version (Weinberg, 2005). In the full version the aim is to identify all cases of disease occurrence (as in the nationwide surveillance of invasive Hib infections in Slovakia). The "light" version is designed to allow generalisations to be made about a whole population based on a limited data set (ECDIS).

Surveillance using specific laboratory tests can be very effective. It is, however, necessary to consider the predictive force of surveillance or a specific test at different times and in different environments. Programme design must consider:

– the actual population at risk,

– the development of preventive measures,

– ensuring appropriate sensitivity and specificity in the system,

– managing the expectations of professionals and the general public (Weiberg, 2005).

Microbiological laboratories possess an overabundance of data and it is practically impossible to pick out clusters of cases, seasonal patterns, changes in the distribution of aetiological agents and developments in resistance. A laboratory computer system (LCS) makes it possible to establish an automated surveillance system for clinical microbiology departments to detect changes in the incidence of microorganisms in relation to different variables. Such systems offer more or less useful ways to implement statistical analyses. Despite the technical possibilities, no laboratory computer system automatically generates reports on changes in statistical characteristics. The first efforts to develop programs extending a laboratory computer system to report at set intervals on comparisons of the incidence of isolated strains and their characteristics in a certain department, with a certain type of material, in a certain hospital were reported in 1992 (Dessau, 1993).

A system can be set up to look for and output information on potential clusters of infections such as gastroenteritis, hospital-acquired infections caused by a specific microorganism, or seasonal epidemics caused by a specific aetiological agent. A system can register the occurrence of unusual microorganisms. The regular provision of information can be used for continual surveillance for potentially serious situations. It can provide extensive data for detailed monitoring.

Most surveillance of infectious diseases is based on laboratory activities to identify or confirm the presence of a certain aetiological agent associated with the monitored disease. The participation of accredited microbiological laboratories is therefore a prerequisite for ensuring the comparability of surveillance. Their activities influence the results of complex surveillance activities and the procedures used in the control and prevention of infectious diseases. One of the fundamental tasks in planning surveillance activities in a microbiological laboratory is to implement simple, accessible, standardised and easy-to-use diagnostic procedures that allow comparable information to be gathered in all levels of laboratories. At the same time, every microbiological laboratory performs continuous non-specific surveillance to detect emerging and re-emerging microorganisms or unusual disease clusters as a first-line unit in the development, application and evaluation of specific intervention measures.

A microbiology laboratory can provide real-time data on infection aetiology and on the susceptibility of bacterial agents to antibiotics. The time gap between sampling and the delivery of examination results means that they are not always used in the selection or modification of a patient's treatment. They are however of vital importance for evaluating diagnosis accuracy, treatment effectiveness and the collection of data on the prevalence of the given infection type and the current resistance levels in the laboratory's catchment area (Hoza, 2005). Such data can be used to track increases in resistance, estimate the time of its development and make changes in therapeutic procedures that reduce rates of unsuccessful treatment and thus reduce the cost of antibiotic treatment.

Microorganisms' resistance to antibiotics has a strongly regional character and the empirical administration of an antibiotic for an acute bacterial infection is admissible provided that the physician's choice of therapy respects the probable agent of the infection and its local level of resistance and it should be verified by a follow-up microbiological examination of the patient (NCCLS, 2003). This makes clear the importance of a microbiological examination of a bacterial infection for verifying diagnosis accuracy and treatment effectiveness, and to obtain data on the prevalence of individual bacterial pathogens and their resistance within the practitioner's catchment area. Regular feedback from microbiological laboratories helps practitioners to choose the most closely targeted antibiotic with the narrowest spectrum of effect for the infective agent, to limits the growth of resistance and keep treatment costs down.

Microbiological examinations and their results can be a source of surveillance data and for some types for laboratory surveillance they play the largest role in confirming disease occurrence based on the aetiological agent. Microbiological laboratories are regularly involved in programmes monitoring the prevalence of certain diseases, resistance levels, hospital-acquired infections and other epidemiologically significant situations

One of the highest priority tasks is to find a multidisciplinary solution to the global problem of ATB-resistance in bacteria that causes diseases of the respiratory organs. Slovakia has an organisation structure in place for antibiotic policy, with central, hospital and regional commissions regulating the use of anti-infectives. The National Reference Centre (NRC) has

established a network of microbiological laboratories for the surveillance of Hib infections. The continuous and coordinated monitoring of bacterial resistance to antibiotics can mitigate adverse developments and ensure an integrated approach to promoting rational use of antibiotics in both outpatient and hospital practice.

5 MICROBIOLOGY AS AN INSTRUMENT OF COOPERATION

Medical microbiology is both a medical and biological discipline concerned with the study of microorganisms, including bacteria, viruses, fungi and parasites, which are of significance for medicine and which can cause disease in humans. It includes the study of microbial disease pathogenesis and the indicators of such diseases in context with pathological and immunological mechanisms. The main areas that the field aims to develop are:

• the diagnosis of the aetiological agents of diseases and the use of knowledge of microorganisms in the prevention and surveillance of communicable diseases and diseases with a significant impact on public health,

• the diagnostic activities of microbiological laboratories.

The activities of a clinical microbiologist in a clinical microbiology department include:

• providing clinical consultation on the examination, diagnosis and treatment of patients with infectious diseases caused by microorganisms,

• cooperating in the development and implementation of programmes to prevent the spread of infectious diseases across the whole field of healthcare.

Alongside their involvement in such fundamental activities, clinical microbiologists often participate in teaching at all levels and in both basic and applied scientific research. Microorganisms can be a factor in the development of disease in any tissue, organ or system of the human body. This is why microbiologists are regularly involved in cooperation and consultation with clinics. Microbiology is a source of knowledge on the origin and significance of pathogens and has contributed to many discoveries in medical science. Knowing the characteristics of infectious agents makes it possible to do research in treatment and prevention including the preparation of vaccines against fatal and severe diseases.

Medical microbiology is also concerned with the pathogenesis of microbial infections and general procedures for the laboratory diagnosis of infectious diseases. Clinical microbiology applies knowledge from medical microbiology in clinical practice and a clinical microbiology department (OKM in Slovak) is a laboratory in a hospital or medical centre that provides microbiological diagnostic services and consultation with clinical microbiologists.

Doctors specialising in clinical microbiology perform several activities of clinical relevance. They participate in daily consultation with clinicians to provide and implement useful microbiological examinations (Bhatacharya, 2010; Gavan, 1978). Topics of consultation include sampling, the transport of samples, the interpretation of finds in stained preparations, the significance of preliminary and final results in the diagnostic process, the antibiogram and its interpretation in selecting an appropriate empirical treatment, and the use of susceptibility testing in selecting a causal treatment. in some larger modern hospitals, this activity is performed by a consultant medical microbiologist (Riordan, 2002).

In a clinical microbiology department, consultation is just one part of the work of a physician-microbiologist alongside laboratory diagnostics, computer processing, analysis, the preparation of reports for insurance companies, quality control and the preparation of reports on activities and antibiotic susceptibility. Nevertheless, consultation on medical microbiology cannot be performed by any other higher education graduate on the staff.

The position of a clinical hospital microbiologist or an equivalent position in a clinical microbiology department must be performed by a doctor with a specialisation in clinical microbiology and clinical experience, or by a scientific researcher. Clinical experience in the diagnosis of infectious diseases can guide the use of the available procedures and the development of new ones to provide a timely and clinically useful diagnosis. A scientific approach raises the standard of diagnostics by applying the latest scientific knowledge in clinical practice, which achieves the ultimate goal of scientific research – the practical application of scientific knowledge (Gavan, 1978).

Regardless of the level of equipment of the laboratory or the activities and qualifications of the other workers there, doctors specialising in clinical microbiology must be the main contact person for clinical microbiology. Their primary task is to provide consultation that links laboratory findings concerning a patient to their medical history and clinical picture so that the tests are interpreted in the way best reflecting reality.

The role of a microbiologist in a hospital setting is to provide important information for understanding disease pathogenesis based on microorganism characteristics, and for choosing the correct therapy based on an understanding of the mechanism of ATB action and the development

of resistance in relation to an infectious disease. It requires a range of knowledge crossing several disciplines of science and medicine; there are only a few fields of medicine with such a broad scope and such an impact on health care. In order to achieve the results expected from them, clinical microbiologists must be able to bring a broad overview and synthesis to case handling. An external microbiological laboratory can also provide consulting services to a hospital, but it has a disadvantage that there is limited potential for direct communication (Bhattacharya, 2010).

A clinical microbiologist's main administrative tasks in a laboratory include the mandatory reporting of aetiological agents, conducting studies, disease surveillance, susceptibility surveys, updating information in the LCS (aboratory computer system), preparing materials for the commission on rational antibiotic therapy, of which they are a permanent member, writing expert guidance and reporting on these activities to their employer, the Ministry of Health, public health institutions and insurance companies.

Many clinical microbiologists also participate in scientific research and academic activity. They are active researchers, using their practical experience to test new knowledge and scientifically investigate random or recurrent laboratory findings. They plan and conduct scientific experiments that are based on long-term observation in clinical and laboratory medicine. Scientific research benefits workers because it contributes to their academic development and serves as a route by which primary research can obtain practical data and the fruits of research can enter practical application.

The education and training of junior doctors is a repeated activity for microbiologists working in a clinical microbiology department. The summer work experience and internships and the residences before specialisation tests that take place in nearly all fields of health care also take place in clinical microbiology departments. The preparation and presentation of lectures at professional forums, seminars and works are also a regular part of clinical microbiologists' professional activities. Training for small groups of physicians preparing for specialisation tests takes place nearly all year round. Specialists in clinical microbiology also provide practical assistance in obtaining and analysing data and the conducting of examinations in accordance with scientific protocols for the purposes of degree dissertations and theses, research projects, publications and presentations (ASCP Task Force, 2008).

The work of a clinical microbiologist also includes additional professional and voluntary activities. Some are necessary for their scientific and academic development, some influence the level of public health on the local or national level and others help to generate resources for developing their laboratory, establish international contacts and raise awareness of the laboratory. Such activities include expert services, paid services, lectures or studies for research institutes, pharmaceutical companies or companies involved in the development or distribution of microbiological diagnostic equipment. This work demands a well-run laboratory that has not only the necessary equipment and staff, but also an appropriate and supportive working environment that provides the necessary conditions for quality, safety and commercial, administrative and financial security. An atmosphere of trust, inclusiveness and collegiality is also beneficial.

A clinical microbiology department's most important function – consultancy - should be available seven days a week, which can be costly and impossible for a private laboratory, which must make a profit besides providing everything described above. Broader coverage is possible in conditions where academic teaching is combined with a clinical microbiology department in a treatment-prevention facility. Activities can be supported by multi-source financing from insurance companies, self-payers, research grants, co-financing of private laboratories by allocating a certain part of revenues for the performance of above-standard, research-oriented and financial demanding activities of clinical microbiology departments). Such an approach supports optimal, modern laboratory services in the patient care process.

In the future, microbiology could become more important than ever if it can stop its incorporation into the system of common examination and treatment units (SVLZ in SLovak) and it can focus on activities in clinical microbiology based on laboratory work. If clinical microbiologists do not engage directly in laboratory microbiology, they cannot influence the scope of activities expected of them. There are many issues – the discovery of new pathogens and the rediscovery of old forgotten ones, the growth of antimicrobial resistance, food safety issues, bioterrorism, the aging population and their susceptibility to common diseases, public health problems associated with global migration – all of which highlight the acute need for specialists in laboratory, medical and clinical microbiology (Thomson, 1995).

Clinical microbiology departments are thriving thanks to privatisation and new molecular technologies for detecting pathogens and antimicrobial resistance. If the two groups of workers in microbiology – doctors and other university-educated microbiologists – come into conflict, the field will cease developing and start falling behind. University-educated microbiologists from outside medicine (laboratory technicians, specialists in laboratory examination methods, workers in large laboratories) bring the field excellent knowledge of diagnostic techniques and the latest technical developments, which can be applied in testing samples and identifying microorganisms. At the same time, they have very limited knowledge of the pathogenesis of infectious diseases and the roles microorganisms play at different stages in their development. A microbiologist who is a doctor may not always fully grasp the technology, but they know the clinical significance of test results. Regardless of whether microbiological findings are produced by conventional or modern methods, they must be correctly interpreted in line with established knowledge taking into consideration other characteristics of the patient and their disease.

Basic and applied research carried out during and through clinical testing has been fundamental to the development of clinical microbiology. Such research has been financed by the commercial manufacturers of laboratory equipment, diagnostic kits and the pharmaceutical industry. Maintaining productive relationships and sustaining clinical research are important aspects of work in a modern, financially successful laboratory, whether private or otherwise, if it wishes to provide high-quality services without being limited by the obligation to make a profit. It is difficult to obtain funds for clinical testing (insurance, self-payers) and the upkeep of a research programme.

Clinical consultation is a vital part of the work of a top microbiology laboratory. Other activities include education, the acquisition of funds via business plans for multi-source research and development funding, the professional development of staff, the provision of clinical, administrative, education and scientific services, and cooperation with clinical staff and laboratories. The overall aim is to develop and implement new technical methods that are well interpreted in clinical practice, and to lobby for feasible improvements in patient care in professional bodies and the legislative process.

Information on the functions of clinical and medical microbiology and the need for experts must be incorporated into classroom learning. Insufficient knowledge and low financial, social and professional attractiveness suppress interest in this branch of science and medicine. New working methods in professional institutions and greater visibility for current microbiologists would help to raise a new generation of microbiologists

1. to conduct their own research into microorganisms' pathogenic mechanisms and immunogenic structures.
2. in the area of diagnostics
    - to develop and implement new diagnostic procedures in microbiology for confirming infections
    - to strengthen microbiologists' contributions to the interpretation of results,
    - to highlight the importance of individual susceptibility to ATB and
    - to improve the targeting of pharmacotherapy to influence the development of microorganisms' resistance to ATB through the effective use of susceptibility surveys,
3. to understand the potential and the activities required of microbiology, microbiological laboratories and microbiologists for epidemiological surveillance programmes,
4. to fulfil microbiologists' role in the upkeep of laboratory work, consultation, the selection of diagnostic procedures and the interpretation of their results in combination with scientific work in other fields of medicine relating to microbial diseases.

The topics of microbiologists' own research include the study of bacteria, viruses, fungi and parasites, their pathogenesis, their susceptibility to antimicrobial therapy and diagnostic procedures. They are also often asked to cooperate in the investigation of samples for the research projects, scientific papers, degree dissertations and theses of colleagues and students in other medical disciplines. They are also consulted on various professional publications, presentations, articles and lectures on the analysis of new, experimental or interesting information on the prevalence, diagnosis and treatment of diseases caused by microorganisms, including new treatment methods. The methodology of research work often requires the participation of a microbiologist. Depending on the character of the work, the microbiologist can contribute the

results of their own scientific research, or their practical experience in innovation, experimentation or the application of knowledge. The microbiologist is therefore often included in a team of authors, cited as a co-author or thanked as part of the acknowledgements. It is rare for a publication on, for example, the incidence of infectious keratoconjunctivitis not to present data on the method used to determine the aetiological diagnosis.

The most common partners and closest collaborators or most faithful clients of microbiologists are infectious disease specialists, paediatricians, epidemiologists and public health doctors. Interesting case reports and evidence of unusual infections (imported infections) can be brought to a successful diagnostic conclusion through close collegial relationships. Such cooperation usually begins during a future microbiologist's pre-attestation period and early work experience in an infectious disease department. While the activities performed there may not seem very relevant to the future microbiologist, the experience is important for establishing direct contacts and getting to know the environment, the challenges and the possibilities of laboratory diagnostics of microorganisms.

Microbiological examinations and serological evidence of specific antibodies contribute to disease diagnosis as part of a broad range of diagnostic procedures; they contribute to identifying a suspected aetiological agent as the cause of disease. Serological tests are useful in differential diagnosis. The selection of identification or serological tests and the timing of sampling requires close cooperation between a microbiologist and an infectious disease specialist to link clinical symptoms to knowledge of stages in the pathogenesis of a specific agent. Parasitic diseases are especially troublesome.

Diagnosis of parasitic infections differs in many fundamental ways from bacterial and viral infections. The reason is that parasitic agents belong to the animal kingdom and most of them have a complex lifecycle with the alternation of two or more hosts. Thorough knowledge of a parasite's biology is therefore essential for understanding the symptomatology, the pathogenesis of the disease and especially its diagnosis and treatment. Where clinical symptoms are absent or ambiguous, laboratory diagnosis is the main and often the only way to obtain evidence of parasitic infection.

In medical parasitology, it is usually very difficult to diagnose a disease based on its clinical manifestations because of their potential diversity. This is why laboratory evidence is a reliable way to determine the correct diagnosis, and often the only way. A specific laboratory technique can provide direct evidence and locate the parasite in the human body. If direct evidence cannot be obtained, indirect methods are used based on evidence of antibodies. A laboratory is able to detect a parasite from biological material during the prepatent period after infection. A doctor may not need to know in detail how the parasite behaves in the human body. It is, however, important for correctly determining the method, quantity, frequency, location and suitable time for the sampling of biological material and the examination. It is a rule of medical parasitology that one examination is not enough, especially if the result is negative. Such infections have what is called a "negative" stage, when the parasite cannot be found in investigated material even though infection persists.

In a time of intensive global migration whether for tourism or for economic, social or professional reasons, there are increasing opportunities for new infectious agents to enter Slovakia or for imported infections to occur in residents, migrants and visitors. Microbiology labs and microbiologists should be prepared to diagnose even uncommon infections, which involves both theoretical readiness and management to ensure the timely availability of diagnostic procedures. Newly emerging infections are often acute and can create a panic. In recent years there has been an increase in the number of foreign nationals coming to work in Slovakia.

Microbiological laboratories are asked to identify aetiological agents in samples from the whole region and the potential number is very large. The information needed to manage an individual patient's illness is unique and depends on the specific disease and its stages. The set of all examinations, or a subset based on strict discrimination criteria, is an important source of information for research and practical activities concerned with monitoring the characteristics of mass outbreaks of diseases or the identification of new properties in the spreading of infectious diseases. For this reason, epidemiologists work very closely with microbiologists.

The framework of measures, procedures and laws for health protection in different areas of human life is studied and overseen by specialists in hygiene and public health. These specialists continuously evaluate the current situation to determine whether the framework is up-to-date, effective or in need of revision. Where communicable diseases have a microbial aetiology, a microbiologist's participation is indispensable for their prevention and prophylaxis, and control of the effectiveness of such measures. A microbiologist cooperates in identifying objective factors in the external environment and determining the role of microorganisms in their changes. The most common areas include monitoring how resistant strains spread to become endemic in the environment, the contribution of contaminated materials and instruments and their handling to the spread of infection and the characteristics of microorganisms that permit them to survive in the environment or colonise staff or patients. A microbiologist is closely involved in the monitoring and characterisation of new microbial properties and in the identification of risks based on the discovery of strains' changing pathogenic potential and virulence, and their mechanisms. In our present ecological, medical and social conditions, such active cooperation is vital to ensure the correct interpretation of the occurrence of emergent and re-emergent microorganisms and microorganisms presenting a potential risk.

6 IMMUNOSEROLOGY OF INFECTIOUS DISEASES

The immunodiagnosis of infectious diseases is currently an area of considerable interest because of the increasing practical application of modern automated examination systems and modules. Specialised immunoserology laboratories in clinical microbiology departments are decreasing in number as they are, for various reasons, incorporated into combined SVLZ laboratories, and sometimes find it very difficult to preserve their identity in the presence of rapid, computer-controlled analysers. Despite the need for standardised methods that eliminate subjective influences and the risk of individual error, and despite the effective implementation of automatic methods that have replaced the manual and repetitive (not thoughtless) work of laboratory personnel, every examination of a biological sample requires an individualised approach in the selection of methods, its timing and above all in its interpretation. We now have automatic systems that can test 800 to 1000 samples per hour for almost any biochemical, haematological, immunological or serological parameter that the doctor providing treatment could indicate – and sometimes more (including molybdenum in tears - a term used for clinically irrelevant laboratory examinations), but the downside of this is the risk of "results overload". This happens when the supply of test results is more than clinicians can use or interpret. Doctors may over-order tests and abdicate their professional insight and expertise because society does not properly recognise their status and they decide to pass the buck. Requesting an examination means more than asking for every test that a laboratory offers. The request for an examination should consider what factors will affect clinical decisions and are in that sense what is worth the investigation and the wait for results. At the same time, there are many routine examination procedures where doctors do not need a long time for reflection because their training, experience and knowledge tell them what they need right away. It is the same as with food. Sometimes it is enough to have a quick bite in the fast food restaurant and sometimes you need to be pampered by top professionals and Michelin-star levels of service.

This textbook aims to present the typical characteristics of immunodiagnosis for infectious diseases, the identification of possible procedures, the conditions that affect the selection of methods and how immunodiagnosis can be used to determine the stage a disease has reached in its progression. The prepared second part will describe in detail the laboratory procedures for

obtaining evidence of the microbial aetiology of selected diseases, and the indication and interpretation of these procedures.

6.1 Principles of immune response of use in serological diagnostics

Bacteria

The immune response to **extracellular bacteria** must deal with all the mechanisms that a microorganism uses to penetrate and invade the human organism and cause disease. A main part of the specific immune response is the production of antibodies against structural antigens (against capsular polysaccharides, surface antigens) or molecules released by living or degraded bacteria (exotoxins, extracellular enzymes). Some antibodies against certain types of bacterial antigens are protective (for example, anti-capsular antibodies against PRP Hib) and their presence in sufficient concentration can prevent the spread of infection, while other are neutralising and prevent tissue damage to the host (antibodies against tetanus ad diphtheria toxins). In most cases they provide diagnostic evidence of the presence of a microorganism or the type of disease caused. Complement activation contributes to successful opsonisation, either in the presence or absence of specific antibodies. The MAC – membrane attack complex – formed on the cell membrane surface as the end product of the complement activation cascade disrupts the membrane structure of some gram-negative bacteria, leading to their lysis (*Neisseria meningitidis*). Complement activation is necessary for the release of chemotactic factors (C5) and their attraction to the site of infection. Endotoxin released from the wall of gram-negative bacteria or present at the site of infection at a time of massive proliferation (*N. meningitidis*) activates the complement cascade by an alternative pathway in the absence of antibodies. It can cause PMNL degranulation and release cytokines with significant biological effects that fulfil the function of non-specific immunity at sufficient concentration. An untreated gram-negative infection or high concentrations of free endotoxin in circulation can lead to potentially lethal effects making up the clinical picture of endotoxin shock.

The main immune response to **intracellular** pathogens is cell-based, described as a Type IV hypersensitivity reaction – the delayed hypersensitivity of T-cells including lymphocytes, cytokines and macrophages. There are two types of approaches to detecting this specific hypersensitivity. A skin test can be conducted in vivo based on the reaction to purified antigens

administered intradermally (tuberculin test) or in vitro with a classical lymphocyte transformation test to which purified antigens are added. Another test that is currently used frequently to test for latent *Mycobacterium tuberculosis* infections in the IGRA (Interferon Gamma Release Assay) test. Great care must be exercised in interpreting both these tests because of the high risk of non-specific false negative results in patients where an intracellular infection suppresses immune responses. If antibodies are generated for an infection with intracellular pathogens, they can have diagnostic significance, but they usually do not perform a protective function and are not indicative of specific immunity.

Viruses

Antibodies (immunoglobulin G - IgG and immunoglobulin M - IgM) are able to bind directly to extracellularly located viruses and their antigenic determinants (epitopes) and to prevent the virus from binding to its target cell. If a virus causes viremia, neutralising antibodies are produced. Two types exist – complement-independent and complement-stimulating. Antibodies of type IgM, IgA and IgG are capable of neutralising the infectious activity of all known viruses provided that they are present at their site of action. Intracellular or vertically transmitted viruses are not affected by the neutralising effect of antibodies (and escape the host's immune system). Antibodies also reduce viruses' infectivity by preventing them from attaching to specific target cell receptors or by making conformational changes in the viruses' structure that promote aggregation. Aggregation increases the effectiveness of antibody-dependent elimination mechanisms such as opsonisation and activation of the complement cascade, or their combination. Hepatitis B is an example of a virus that can be eliminated by immune-mediated antibodies at the time of their release into the blood from the target tissue where it proliferates (secondary viremia). There some infections where antibodies against viral proteins can have an immunopathological effect. For example, if serum antibodies against respiratory syncytial virus (RSV) are not protective and represent passive immunity transferred through the placenta from the mother, they can cause an immune complex disease – a type III hypersensitivity reaction, Arthus Reaction – in the lungs of a new-born baby with a postnatal RSV infection. A similar effect of preformed antiviral antibodies has been observed when a patient becomes re-infected with the measles virus or is infected with a wild-type measles virus after being vaccinated using an older type of live measles vaccine. The immune response to intracellular and vertically

transmitted viruses usually involves cell-mediated cytotoxicity. Cytotoxic effector cells recognise changes in surface antigens caused by viral infection. Endogenously altered antigens are displayed on the infected cell's surface by the MHC I molecule, where they attract specific T lymphocytes or activate non-specific cytolytic natural killer cells and macrophages. Antibody-dependent cell-mediated cytotoxicity (ADCC) is another effective mechanism of antiviral cytotoxicity.

Fungi

In diseases caused by pathogenic and medically significant fungi (yeasts and moulds), the primary immune response is cell-mediated. When certain fungi cause system-wide effects or fungemia, the detection of specific IgM and IgG antibodies can contribute to their diagnosis. These antibodies have no protective effect, however. They are most frequently demonstrated by immunoprecipitation.

Parasites

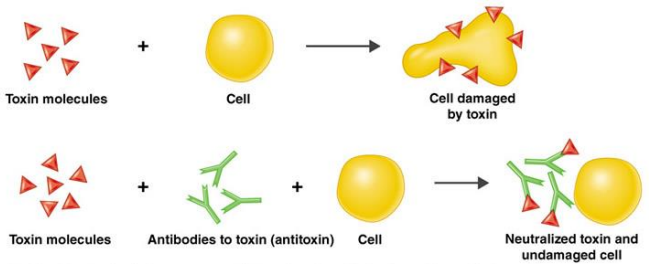
Parasites (protozoa and worms) cause varied immune responses. Worm infections are characterised by the prevalence of IgE antibody production. Worms specifically stimulate CD4+Th1 - T helper lymphocytes, which produce IL-4 a IL-5**.** Antibody-dependent cell cytotoxicity (ADCC) via eosinophils and IgE is considered effective for the elimination of worms because the major basic protein in eosinophil granules is toxic for worms. In this case the ADCC mechanism is that specific IgE antibodies attach to the worms and the IgE molecules' Fc fragments bind to eosinophils, which destroy the worms. Some parasites lay eggs that induce granuloma production (e.g. *Schistosoma mansoni*) in some organs, e.g. the liver. Stimulated CD4+T lymphocytes activate macrophages, which form granulomas to isolate the eggs from other tissue. Fibrosis formation interrupts venous blood flow in the liver, leading to hypertension and cirrhosis. Intracellular protozoa often activate specific cytotoxic T cells. It is a key point for preventing the dissemination of intracellular localised plasmodium parasites in malaria. Immune complexes formed by antibodies and parasite antigens can be caught in the narrow vessels of the kidneys and cause immune complex glomerulonephritis. Animal parasites have evolved remarkable defence mechanisms that can lead to chronic infections, especially in vertebrates.

Natural defences against parasites are very weak and ineffective. Parasites have evolved highly sophisticated mechanisms for evading their hosts' adaptive (specific) immune systems.

6.2 Basic terminology for laboratory evidence of microbial disease aetiology

Direct diagnosis means making visible or proving the presence of an aetiological agent or some of its components. This can be achieved by various methods, which are selected depending on the type of suspected microorganism and the stage of the disease. These methods include microscopic and cultivation methods for bacteria, fungi and parasites, evidence of antigens (based on the serological and immunodiagnostic methods described below), nucleic acid evidence (PCR methods), protein identification (Malditoff, proteomics), virus isolation and evidence of pathogenic viral properties (cytopathic effect, interference, neutralisation).

Indirect diagnosis – involves the detection of specific antibodies of various isotypes (IgG, IgA, IgM) and functions (neutralising, complement fixation) which are produced by a host in different quantities at different times in the immune response to infection. An older name, serological reactions, refers to the medium in which the antibodies are found. The modern name, immunodiagnostic methods, emphasises the antibodies' role as part of the immune system. Laboratory procedures using **serological reactions** or immunodiagnostic approaches are always based on the reaction between an antigen and a specific antibody in a certain medium.



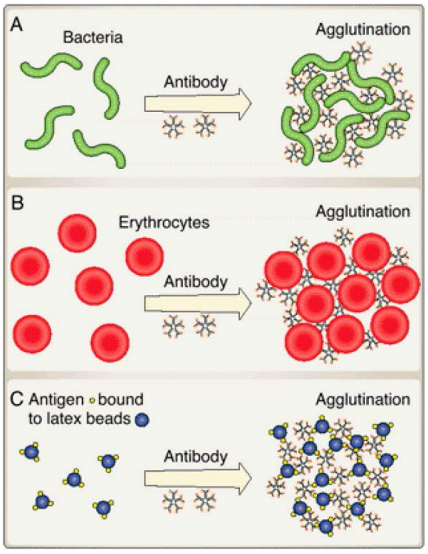
Neutralisation *– antibodies bind to toxins, occupying the antigenic structures and preventing them from binding to specific receptors on target cells and neutralising their toxicity.*

The components of a serological reaction can have different characteristics, which determine their use:

- **antigens** can be corpuscular, soluble, electrophoretically or otherwise separated and fixed, recombinant...
- **antibodies** can have different isotypes or be total, monoclonal, protective, neutralising ...
- The **medium** in which the reaction takes place determines the basis for visualising the result and the diversity of physical and chemical properties of media requires a diversity

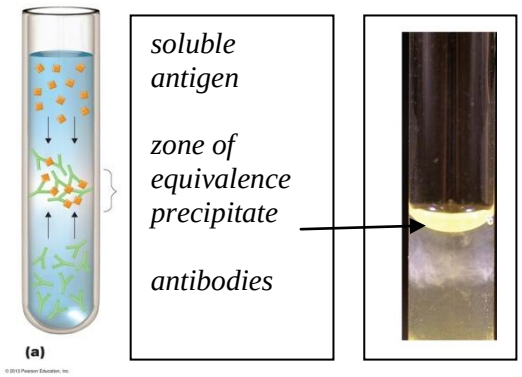
of diagnostic methods and procedure, each of which has its justification and precise indication.

To establish evidence of antibodies against a corpuscular antigen such as bacteria in a liquid medium, agglutination methods are used. The specificity and sensitivity of methods can be increased by binding the antigen (or antibody) to another material, which is the basis of the hemagglutination or latex agglutination methods.

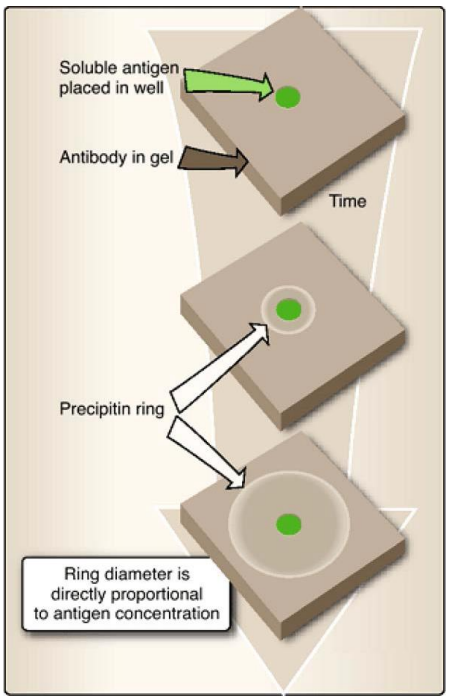


Antibodies can bind to antigens on cells or particles and bind them together – agglutinate them. ***Agglutination*** *works by catching microorganisms in a molecular net, which inhibits their movement and makes them more susceptible to destruction. Antibodies can bind with infectious agents (**A**), host cells such as erythrocytes (**B – hemagglutination**) or an inert material such as latex (**C – latex agglutination**), when specific antigens are expressed on their surface.*

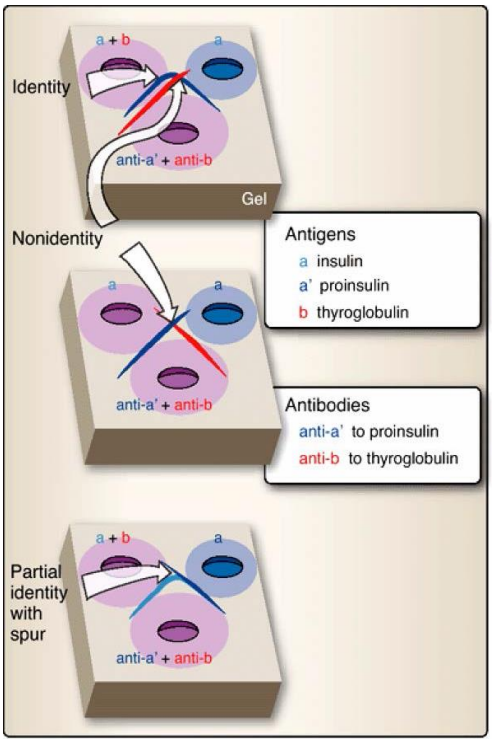
Evidence of antibodies against soluble antigens (e.g. toxins) can be obtained from a liquid or semi-solid medium (agar), where the soluble antigen diffuses and precipitates upon encountering a specific antibody. Precipitation reactions are carried out in a liquid medium that is layered with the patient's serum containing antibodies to the antigen in the solution.



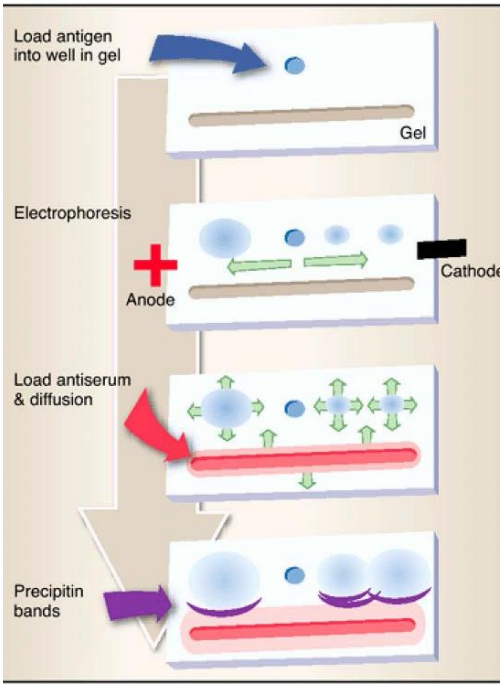
The techniques of agar gel immunodiffusion are highly developed and the most common variants currently in use include radial immunodiffusion, double immunodiffusion and crossed immunoelectrophoresis. The methods are often known by the names of their inventors (Mancini, Ouchterlony).



Radial immunodiffusion (Mancini method) *is a technique based on the diffusion of a soluble antigen through an agar gel containing an antibody. A layer of liquid agar containing an antibody is poured onto a slide and then allowed to solidify. The antigen is then placed in a well cut into the gel and diffuses radially into the gel mass. A precipitin ring forms in the zone of equivalence. The diameter of the precipitin ring is directly proportional to antigen concentration and comparison with standard concentrations and the creation of a standard curve makes it possible to determine the exact concentration of the antigen. The method can be used to quantify serum proteins or to identify antibodies against various microbial antigens in experimental research.*



***Double diffusion (Ouchterlony method)**. This is a modification of radial immunodiffusion. Wells are cut into a solidified agar gel. Soluble antigens are placed in one well and antibodies in another, usually in the centre, from where they diffuse into the gel. **Top panel**: A precipitin line is formed in the zone of equivalence. The red precipitin line forms between the antibody well and top well on the left containing 2 antigens a+b because the serum contained antibodies against both antigens (anti-a and anti-b). The shape of the blue precipitin line for the anti-a antibody is determined by the fact that the top right well contains only antigen a. The illustration shows identical antigens in two wells **Middle panel:** detection of two non-identical antigens with two types of antibodies, each against one of the antigens (non-identical antigens). **Bottom panel**: The antibodies react against two similar antigens. The blue precipitin line indicates partial identity with a non-specific spur.*

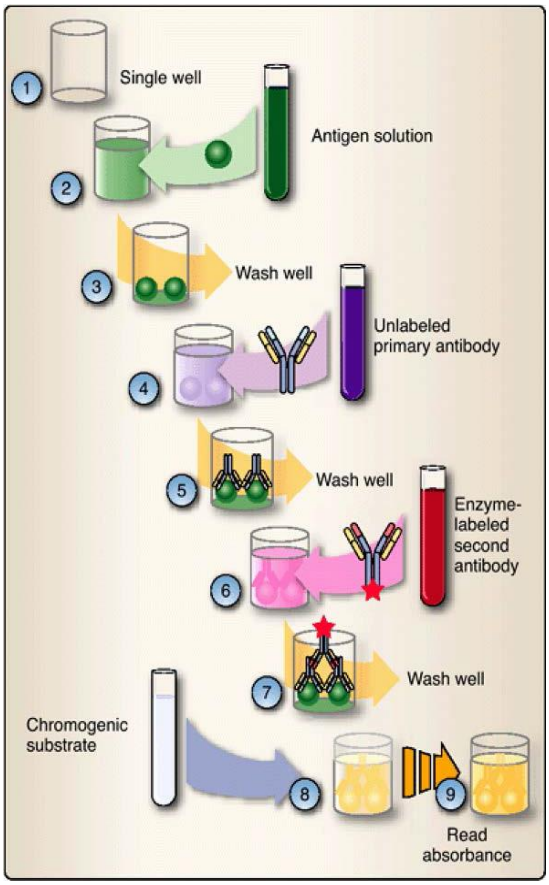


***Immunoelectrophoresis** is a modified form of double diffusion. An antigen is placed in a well in an agar gel. A current is applied to the gel causing the migration of antigens influenced by charge and size. An opening is then cut in the gel and filled with antiserum containing antibodies. Both antigens (blue) and antibodies (red) diffuse into the gel and create precipitin lines (purple) in the zone of equivalence.*

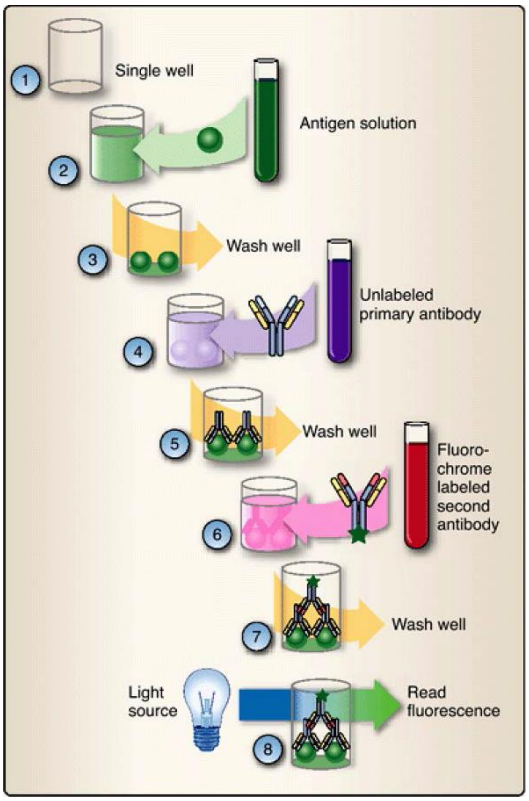
Recombinant antigens – some microorganisms, such as *E. Coli (Escherichia coli)* bacteria, are used in the preparation of human or other antigens. The technique exploits bacterial cells' capability for heterologous gene expression. By incorporating the genetic information for a

required antigen into the *E. coli* genome, the foreign genetic material can be transcribed and translated to produce the antigen. A task in the process is purifying the desired antigen from *E. coli's* own antigens, which could produce false positive results in tests using the recombinant antigens. To modify a famous Slovak saying, we are not rich enough to buy cheap diagnostic sets.

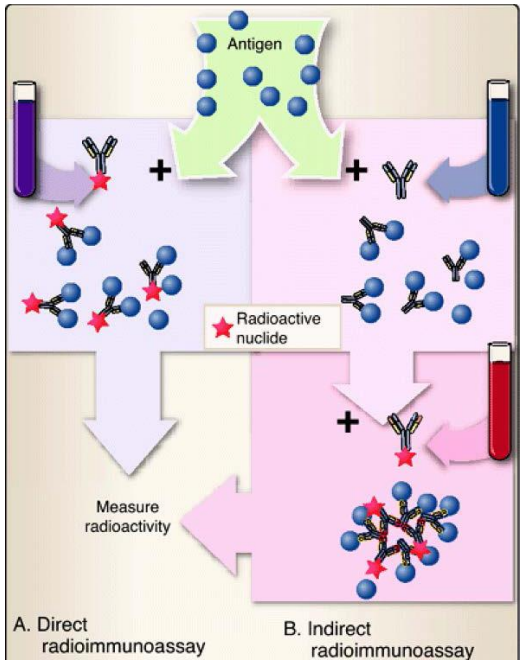
If the capturing molecule or structure, whether antigen or antibody, is bound to a solid phase – structure, the immunocomplex formed by the reaction of the antigen and antibody can be made visible by a diagnostic antibody – conjugate – which is labelled with an enzyme, radionuclide or fluorochrome. This set-up is the basis for the ELISA, RIA and immunofluorescence assays, which allow quantification by detecting the intensity of a labelled antibody.



*In **ELISA - enzyme-linked immunosorbent assay**, enzyme-labelled antibodies are used to identify a specific epitope (antigen). 1. The test is conducted in well on a polystyrene microtiter plate (usually in a set containing 96 such wells) capable of adsorbing protein. 2. A soluble antigen is added and covalently bound to the artificial surface of the well. 3. Unbound material is removed by washing. 4. Serum containing antibodies is added to the well. Specific antibodies bind strongly to the antigen 5. Unbound antibodies are removed by washing. 6. An enzyme-labelled antibody that binds to human antibody molecules is added to the well. 7. The unbound enzyme-labelled antibodies are removed by washing. 8. A chromogen substrate is added to the well. 9. The change in colour shows the presence of the enzyme-labelled secondary antibody that is bound only if the specific antibodies against the epitope are present in the patient's serum. The intensity of the colour change shows the quantity of the detected epitope.*



Fluorescent Immunoassay (FIA) *has the same purpose as ELISA. 1. The test is conducted in well on a polystyrene microtiter plate (usually in a set containing 96 such wells) capable of adsorbing protein. 2. A soluble antigen is added and covalently bound to the artificial surface of the well. 3. Unbound material is removed by washing. 4. Serum containing antibodies is added to the well. Specific antibodies bind strongly to the antigen 5. Unbound antibodies are removed by washing. 6. A fluorochrome-labelled antibody that binds to human antibody molecules is added to the well. 7. The unbound labelled antibodies are removed by washing. 8. Fluorescence indicates the presence of the epitope.*



Radioimmunoassay (RIA). *As the name suggests, this assay uses a radionuclide such as* I^{125} *to label the primary or secondary antibody or antigen.* ***A.*** *Direct RIA uses radionuclide labelling of the primary antibodies which are incubated with the antigen. The unbound antibodies are washed off and the radioactivity is recorded by a gamma counter.* ***B****. The indirect RIA method first applies unlabelled primary antibodies that bind to the antigen and then a secondary radionuclide-labelled antibody that binds to immunoglobulin. A gamma counter measures the bound radioactivity.*

6.3 Characteristics of specific elements of immunodiagnostic procedures

Immunodiagnosis is an approach to diagnosing a patient's illness based on the detection of antigens or antibodies from which the infectious agent or the components of the immune response to infection can be identified.

There are two main approaches within immunodiagnosis.

- detection of specific antigens or
- the determination of the antigens of specific antibodies.

As stated earlier, the detection of specific microorganisms or their parts (antigens) in a patient's biological sample is the essence of the **direct diagnosis** of the microorganism or the determination of its antigenic properties, also known as their serotyping.

To determine the cause of a disease by **indirect diagnosis** means identifying the antigen of specific antibodies or individual classes of specific antibodies, which are molecules of individual isotypes of immunoglobulins.

Each detection of specific antibodies by a certain method has its own evidential value. The classical methods (agglutination, CFR, precipitation etc.) detect a mixture of antibody classes and diagnosis requires observation of the dynamics of their production in at least samples taken over 14–21 days. Different stages of disease are characterised by the presence of specific antibodies of different isotypes.

Immunoglobulin M (IgM) antibodies appear at the start of infection and are detectable from the 7th to 10th day after infection, depending on the sensitivity of the assay. They are a sign of acute infection but remain for 3–6 months after primary infection. In a new-born infant, it is a sign of intrauterine infection. Because the presence of IgM in serum is temporary, a find indicates a recent acute infection and it can usually be confirmed with one sample. IgM antibodies do not occur exclusively with primary infection and there are cases where they persist for longer. The detected IgM antibodies must be evaluated for each infectious agent individually.

Immunoglobulin G (IgG) antibodies appear later (around the 14th day of infection) and culminate in the 4th to 6th week after infection. They persist for a long time and can maintain a long-term to lifetime presence (depending on the primary antigenic stimulus, the patient's immune status and subsequent re-exposure with a booster effect on antibody concentration). They are a sign of protective immunity. Immunological tests for IgG antibodies sometimes require paired samples.

The first sample should be taken during the acute phase of infection and the second during the convalescence period, as in classical serological methods (CFR, indirect agglutination).

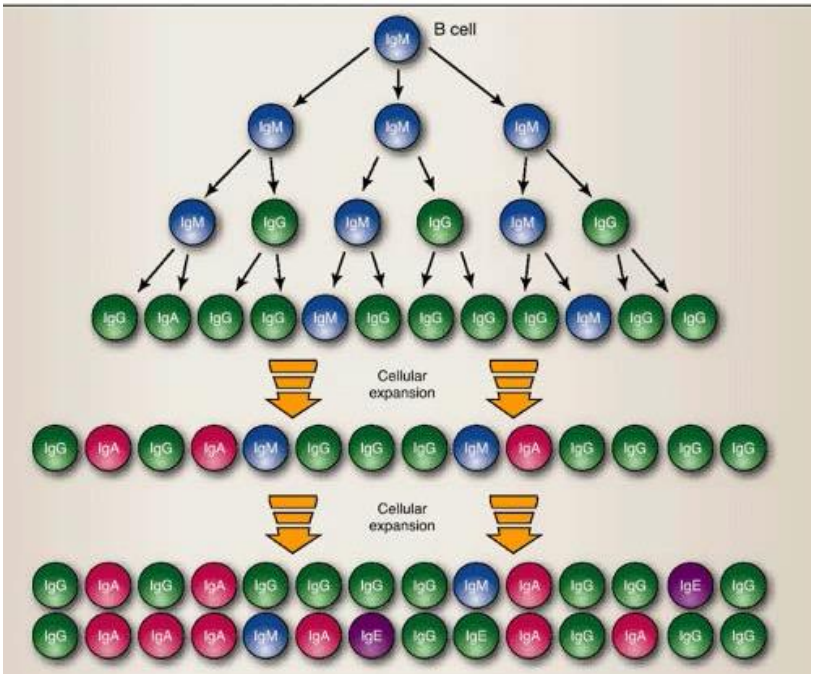
Serum IgA antibodies have varying diagnostic values for different aetiological agents. In general, IgA antibodies are produced in the acute phase of the disease, but they are also produced when a chronic disease is reactivated or on re-exposure to an infectious agent.

IgE antibodies are associated with parasitic diseases. The diagnostic significance of IgD antibodies is unclear. They appear mainly in connection with the presentation of an antigen and the formation, activation and stimulation of the specific immune response.

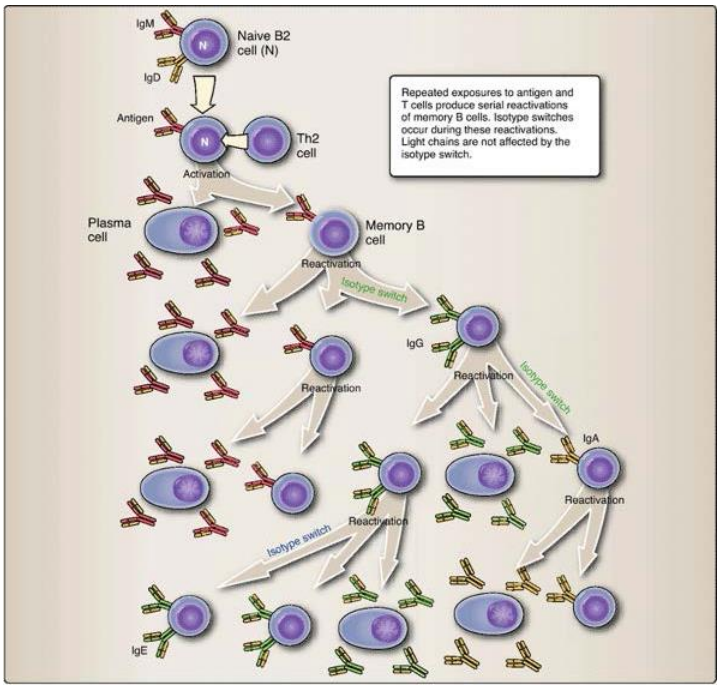
the interpretation of laboratory findings requires experience and good theoretical knowledge because the persistence of antibodies for many years after acute infection and the existence of chronic infections that can become reactivated broadens the range of possible laboratory findings.

Schematic interpretation of findings of individual specific isotypes

Stage of infection	**IgM**	**IgA**	**IgG**
Acute infection	+	**- (+)**	-
Post-acute phase, convalescence	+	**+ (-)**	+
Anamnestic antibodies, **overcome infection, post-vaccination antibodies**	-	-	+
Re-exposure to causal agent **Re-activation of chronic infection**	-	+	+



*The **humoral antibody response** can have a variety of forms and can gradually produce various isotypes of molecules with the same epitope – the same specificity (IgM, IgG, IgA, IgE).*



*Repeated exposure to an antigen produces memory B cell reactivations during which immunoglobulin **isotype switches** take place. IgM changes to IgG, IgA and IgE.*

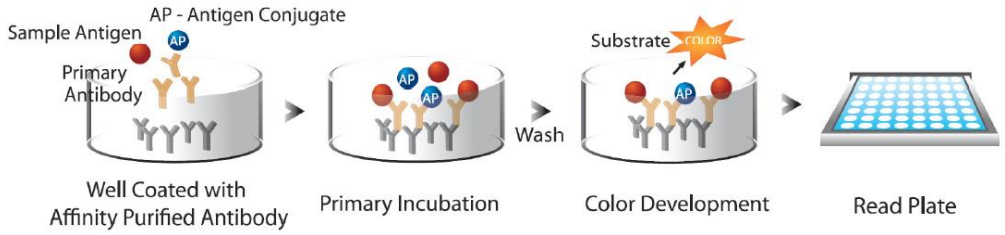
6.3.1 Antigen detection

SPIA – <u>solid phase immunoassay</u> – is the term commonly used in the English literature for tests in which a target molecule is immobilised onto an insoluble substrate. There are three methods

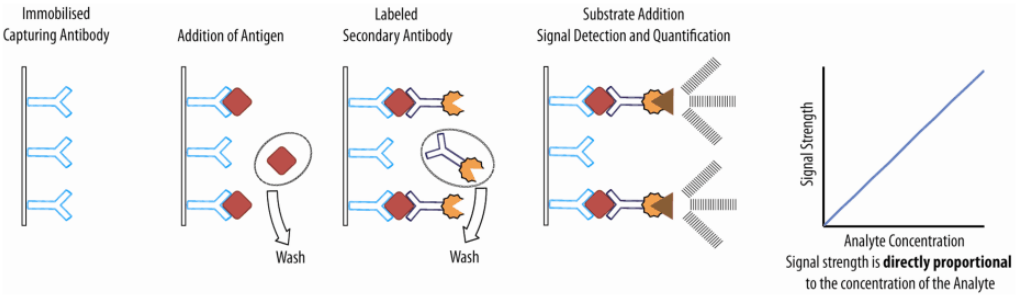
for using such systems to detect antigens: competitive assay, direct sandwich assay and indirect sandwich assay.

In a competitive assay a labelled antigen is mixed with a biological sample that may contain the antigen and this mixture is added to the solid phase (a well in a plate) Both antigens compete for the free binding sites on a limited quantity of antibody immobilised on the solid phase. A negative control contains only the labelled antigen. The difference in activity between the control and the sample is measured. Detection antibodies are labelled with an enzyme.

www.enzolifesciences.com

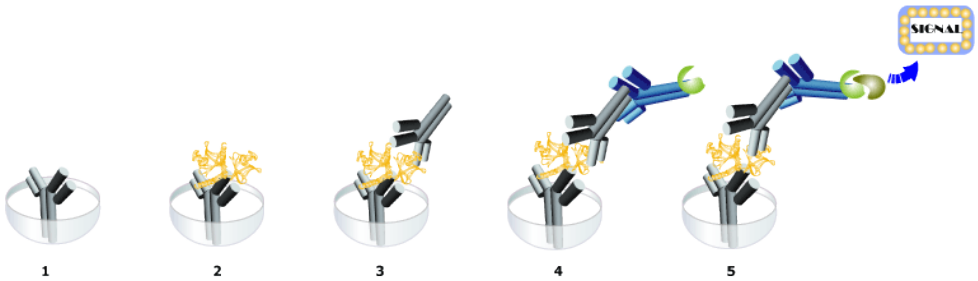


In the direct sandwich (double antibodies) method, a clinical sample is added to immobilised capturing antibodies. Unbound antigen is removed by washing. Enzyme-labelled detection antibodies are then added. The activity of the product after the addition of a substrate is directly proportional to the amount of labelled antibody bound to the antigen on the solid phase. (Unbound labelled antibodies were removed by washing). Assays using polyclonal capturing antibodies and monoclonal detection antibodies give the best results.



The indirect sandwich (double antibodies and antiglobulin antibodies) method also uses capturing antibodies and detection antibodies but they are not labelled with enzyme. Another addition is an

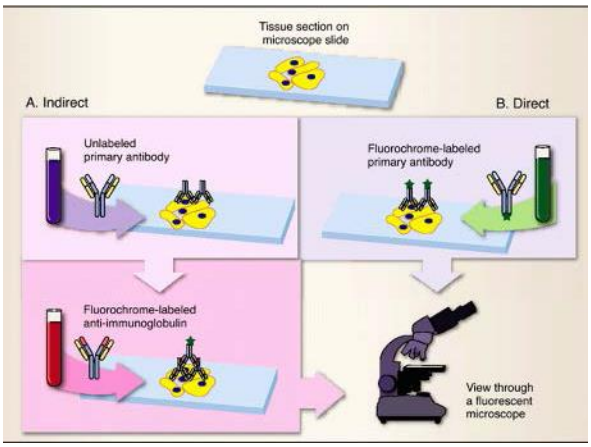
indicator, which is made up of enzyme-labelled antiglobulin animal antibodies that bind with the detection antibodies. The commercial availability of enzyme-labelled antiglobulin animal antibodies makes this system especially popular. It is a very sensitive procedure, but the antisera against various species can be a source of non-specific cross reactions.



www.genwaybio.com

Immunodot (IDA) – this is a similar method to the Western Blot (WB) method used to confirm specific antibodies, but for antigen detection. Both are conducted using a nitrocellulose membrane, which serves as a sold phase of the assay. The antigen in the clinical sample is applied to a nitrocellulose reaction strip on which antibodies are immobilised. Immune complex dot assay is a modified form of the immunodot assay in which the antigen and antibody are made to react and the resulting complex is applied to nitrocellulose and detection is carried out using colloid-labelled animal antibodies. It provides more sensitive detection of antigen in a clinical sample.

Immunofluorescence methods involve the washing and application to a slide (solid phase) of cells from a clinical sample that is thought to contain a microorganism. After immobilisation, fluorochrome-labelled antibodies are added (in a direct immunofluorescence assay – IFA) or antibodies are added and then fluorochrome-labelled anti-immunoglobulin antibodies (in an indirect IFA). If multiple fluorescent dyes are used to identify multiple specific antibodies with different colours, it is possible to identify several antigens in a sample on a single slide. The indirect IFA method allows multiple antigens to be identified in a sample by using different capture antibodies and the same fluorochrome-labelled anti-immunoglobulin antibodies. This procedure is used in virology if several sample preparations have to be prepared (detection of respiratory viruses from nasopharyngeal fluid).

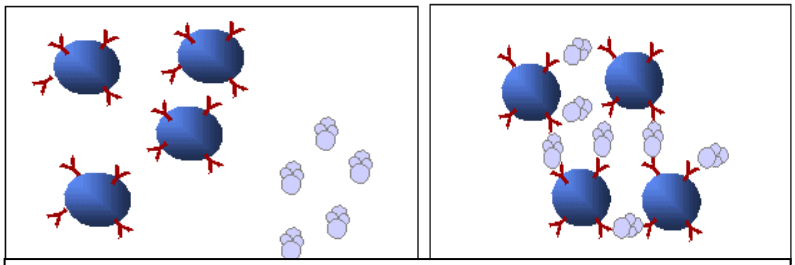


*Immunofluorescence uses a fluorescent dye that binds covalently to antibodies. Thin immobilised slices of tissue are installed on a slide to which is applied a solution containing labelled antibodies (**Direct immunofluorescence, B**) or a solution containing primary antibodies followed, after washing, by fluorochrome-dyed anti-immunoglobulin antibodies (**indirect IF, A**). The presence of epitopes is visualised using a fluorescence microscope.*

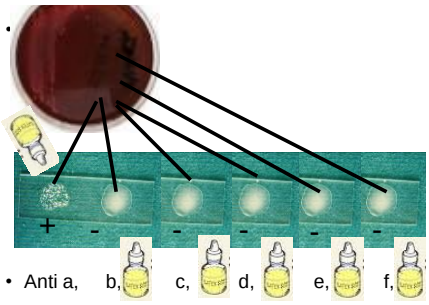
In **immunoelectron microscopy (IEM)** the clinical specimen is embedded in a suitable chemical substance. Thin sections are then applied to a grid and dyed using specific antisera (serum containing a specific antibody) and colloidal gold-labelled anti-immunoglobulin antibodies by applying a drop of the reagent to the section embedded in paraffin. Another possibility in IEM is to use an electron microscopy grid coated with specific antibodies for the capture of specific antigens.

Immunohistochemical staining is based on the histological preparation of a tissue sample whose final steps are the addition of specific antibodies, enzyme-dyed animal antibodies and a suitable substrate for the detection of an antigen in situ in the tissue.

Agglutination – is a classical method that can use specific antibodies in solution (direct agglutination) or antibodies bound to latex particles for detection of a microorganism (latex agglutination) or its antigenic structure (slide agglutination). An antigen in a biological sample reacts with specific antibodies to form non-dispersible clumps while simultaneously clarifying the liquid medium or suspension. It is used for the rapid diagnosis of the causal agent of meningitis by detection in CSF (cerebrospinal fluid) or soluble antigens in urine. Low sensitivity is a disadvantage of such methods. They depend on monitoring a visible antigen-antibody complex. This is usually enough to detect bacterial antigens but no virus antigens, which are smaller and require a larger antigenic mass to create visible clumps.



Latex agglutination *– latex-bound specific antibodies capture microorganisms in the sample*



Serotyping *– By using antisera that are specific for individual antigens, it is possible to determine the antigenic structure of a bacterium.*
A colony of Haemophilus influenzae is tested by antibodies against possible capsular antigens (a, b, c, d, e, f). Positive agglutination takes place only with antiserum a. This determines the bacterial serotype.

6.3.2 Antibody detection

Competitive assays can provide evidence of human antibodies against viral and bacterial (microbial) antigens through the reaction of a sample with a precisely determined quantity of a conjugated specific antibody and incubation of the mixture with a solid phase on which an antigen has been immobilised. If specific antibodies are present in the sample, they will compete with the conjugated antibodies for binding sites on the solid phase. The size, intensity or activity of the resulting reaction is indirectly proportional to the quantity of antibody in the sample. Antibodies against antigens can be prepared in animals or purified from human serum, or monoclonal antibodies can be used. Conjugate preparation requires serial conjugate titration and serial antigen titration to produce a conjugate-antigen pair capable of generating, at the highest dilution, a signal that is reduced if an antibody is present in the sample. An advantage of competitive assays for measuring antibodies is that it is straightforward to prepare specific antibodies, monoclonal and conjugated antibodies. It is easier to purify antibodies than antigen and even a relatively impure antigen can be used for solid phase binding. Competitive assays are more sensitive than indirect assays. The solid phase is usually coated with antigens or anti-immunoglobulin antibodies (anti-total or anti-IgG, IgM, IgA).

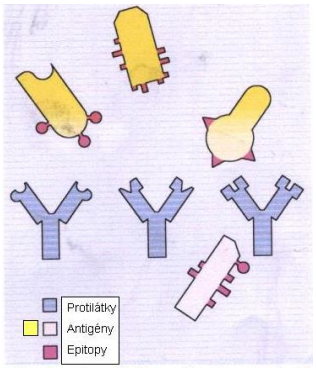
If the solid phase is coated with antigen, the full range of antibodies are captured, otherwise antibodies to human IgA, IgM or IgG from animal sera can be used for detection. Such a procedure requires adsorption of rheumatoid factor, however. If the solid phase is coated with anti-immunoglobulin antibodies, the most frequently used are IgM antibodies. This procedure captures only IgM, which will then be available for the detection labelled antibodies. The next step is the addition of the unlabelled antigen, which is then detected by the detection antibodies. In detecting specific IgG, IgM RF (rheumatoid factor) is also used to coat the solid phase. A sample with specific IgG is mixed with a labelled conjugate and incubated with the solid phase with IgM RF. Only the specific IgG molecule binds to the antigen and the solid phase so only one step (one incubation) is needed. On the other hand, this places high quality requirements on the antigen and IgM RF.

IgM detection provides evidence of acute infection. The most common detection technique is a solid phase immunoassay with an immobilised antigen and secondary IgM specific detection antibodies. False positive reactions are common when IgM RF is present in the patient's serum. False negative results can occur because of competitive inhibition of IgM binding in the presence of high levels of specific IgG. These problems can be solved by removing IgG from the patient's serum by adding precipitating anti-IgG antibodies or by removing RF using an RF sorbent (aggregated IgG).

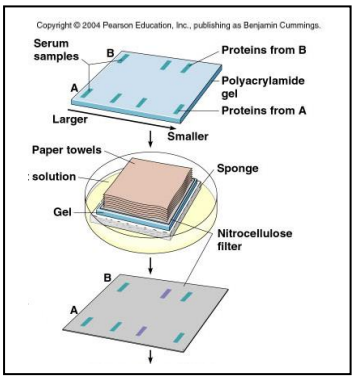
The use of an IgM capture test eliminates certain problems. Polyclonal anti-IgM antibodies are bound to the solid phase and during incubation with the patient's serum, all the IgM binds to the solid phase. The test antigen is then added, which binds to the specific antigen. Labelled secondary antibodies identify the patient's IgM. In this setup, all the IgG antibodies are removed by washing, which eliminates the possibility of a false negative result. A false positive result is possible if IgM RF binds with an IgG conjugate or with any IgG in the sample. This can be avoided by using Fab conjugates or by a direct technique using a labelled antigen in step 2, by which RF-binding immunoglobulins are eliminated. There is still a problem with boundary and low-positive results.

Immunoblot – the molecules forming the antigens and antigenic determinants (epitopes) of a microorganism can be separated via polyacrylamide gel electrophoresis and transferred to nitrocellulose. This is the solid phase for the reaction. The patient's serum, urine or saliva is

applied to the solid phase and after the reaction and staining, it is possible to identify antibodies against a particular antigen of a microorganism, providing more specific results.



*After **separation by electrophoresis**, a microorganism's **antigens** form a visible band containing multiple antigen molecules. The antigens are then transferred to nitrocellulose. The patient's serum is applied and individual antibodies against all antigens are localised at the site of the*



Western blot uses an enzyme-labelled conjugate to identify a created immunocomplex.

Recombinant Immunoblot assay (RIBA) – uses recombinant antigens.

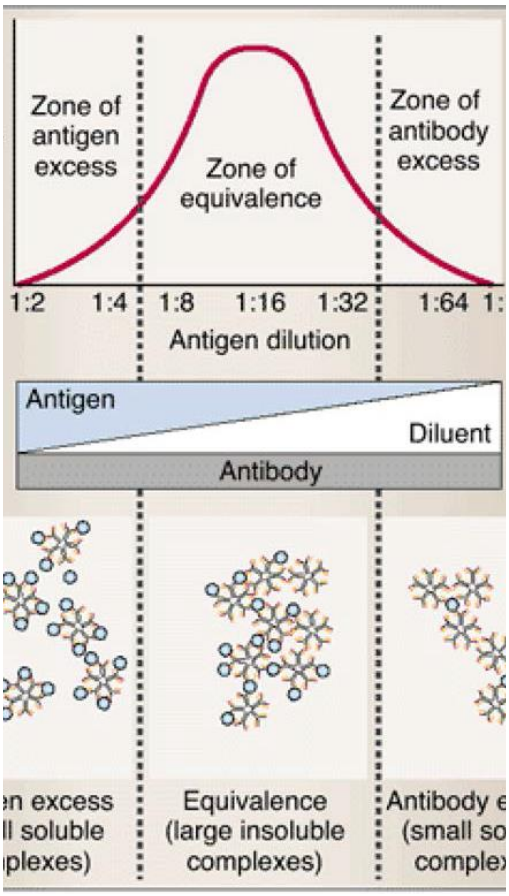
Radioimmunoprecipitation is used in assays to identify specific antibodies through the immunoprecipitation of a microbial protein labelled with a radionuclide that can be detected by scintigraphy and autoradiography. This method, like the other methods using radionuclides is not used in practice. It found its purpose in confirmatory tests.

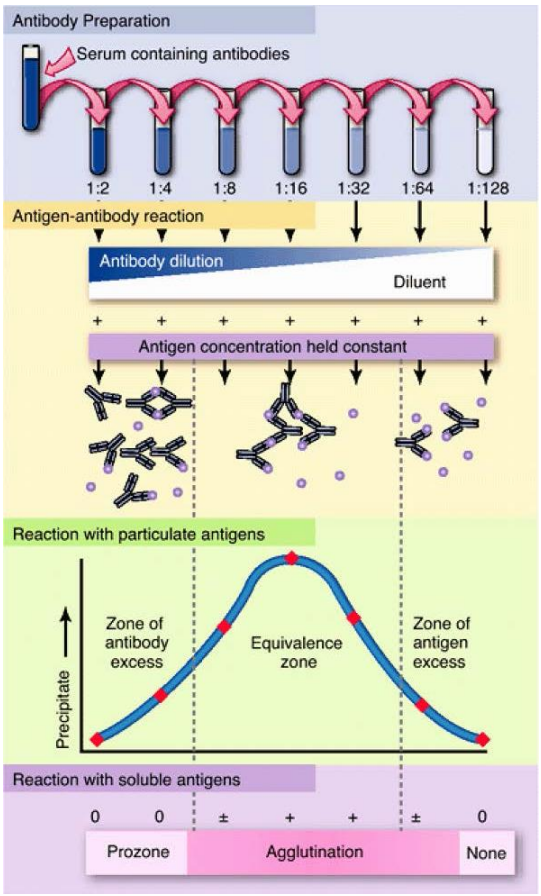
Immunofluorescence – indirect immunofluorescence is also used to detect specific antibodies in patient serum. For this purpose, an antigen is applied to a slide, allowed to dry and fixed with acetone or methanol, after which a (suitably diluted) test serum is applied. Fluorochrome-labelled anti-immunoglobulin antibodies are applied to the slide and after incubation and washing the sample is coated in buffered glycerol. The detection system is viewed using a fluorescence microscope. This method is suitable for detecting antibodies in cases such as parasitic infections (malaria, trypanosomiasis) where the level of antibodies is high because the disease is chronic. Microscopically detected fluorescence is subjectively evaluated and its activity decays gradually. Newer systems support machine reading of fluorescence and the quantification of intensity. In fluorescence microscopy it was possible to provide quantified results by diluting the serum and recording the titre at which the fluorescence was still detectable.

Automated fluorescence readers maintain the solid phase principle in which the antigen is immobilized on a nitrocellulose strip which is immersed in serum and the specific antibodies, if present, bind to the antigen on the stripe. The strip is then immersed in a solution of

fluorochrome-labelled anti-IgG antibodies. The test result is measured by a fluorometer. This is a technically straightforward method that is used in screening tests. A disadvantage compared to fluorescence microscopy is that it is not possible to see the shape or type of fluorescence.

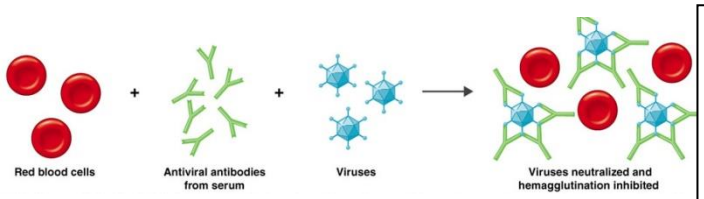
Agglutination – indirect assays are used to detect antibodies in a patient's serum. To this is added an antigen that binds the antibodies for which the sample is being tested. If they are present, a non-dispersible clump will be formed. Agglutination antibodies are total. When setting up the test it is necessary to create the zone of equivalence so that the amount of antigen added to the serum corresponds to the number of antibodies. Otherwise there is a risk of a false negative result and the prozone phenomenon. The zone of equivalence is determined by dilution of the serum and antigen. Agglutination reactions can also be evaluated quantitatively. A positive result indicates the highest serum dilution in which the agglutination is detectable (e.g. 1:128). The result can also be expressed as a titre, which is the inverted serum dilution value (titre 128).





*Serum containing antibodies is diluted in a **geometric series** with each consecutive tube containing half the antibody contraction of the previous tube (1:2, 1:4, 1:8, 1:16...). Antigen-antibody reactions: A multi-epitope antigen is added at a constant concentration to the diluted serum (antibody solution) and an antigen-antibody reaction occurs if the antibody has specificity for the antigen. There are three possible reaction zones: In the zone of equivalence, the concentrations of the antigen and antibody are equal and create the maximum number of immune complexes (agglutinates, precipitates...). In the zone of antibody excess, there is insufficient antigen to create a visible number of immune complexes. Excess antibodies (in a highly positive sample) prevent particle crosslinking and the result appears to be negative (a false negative). In the zone of antigen excess, there are insufficient binding sites and it is not possible for a sufficient number of immune complexes to form. This accounts for the importance of precision in the reaction settings and serum dilution.*

Passive hemagglutination and hemagglutination inhibition assays are modifications of the agglutination test that are used to detect antibodies against both viral and bacterial antigens (*Treponema pallidum*, rubella virus, influenza, RSV). The hemagglutination inhibition test detects the presence of antibodies based on the inhibition of hemagglutination, which some microorganisms cause spontaneously. The highest dilution in which hemagglutination is not visible determines the antibody titre in the tested serum.



*A virus normally causes hemagglutination of red blood cells. If antibodies against the virus are present, they neutralise its function and inhibit hemagglutination – **hemagglutination inhibition test, neutralisation test**.*

7 CONCLUSION

The theoretical part of the present work analyses the field of microbiology. It defines the basic concepts including microbiology itself, and clinical, laboratory and medical microbiology. It presents the role of a microbiologist in the treatment and prevention of illness. This is performed by clinical microbiology departments integrated into the healthcare system. In Slovakia, scientific research is conducted mainly in scientific and academic institutions. This brings scientific work in microbiology into the educational sector. The work describes the significance of the field and its potential contribution to research and diagnosis through cooperation with other fields of medicine and the establishment of a surveillance system in laboratories. It situates clinical and laboratory microbiology within the current healthcare system.

Medical microbiology, which is based on lab work in clinical microbiology departments and the scientific potential of the faculty, has a broad scientific and diagnostic scope and plays a key role in surveillance and other areas of cooperation. These characteristics make microbiology one of the most interesting areas of medicine, with potential applications in science, routine diagnostics and interdisciplinary cooperation in clinical medicine and public health.

- In the area of scientific research, it is necessary to establish and maintain a permanent high level of expertise and material and technical equipment in laboratories in compliance with requirements for accurate lab work and accreditation.
- The education and healthcare systems provide limited opportunities for financing research leading to high quality publications, and grant funding is unpredictable. Multi-source financing of laboratory research in elite institutions is one possible solution, although this requires cooperation with teaching hospitals, insurance companies, for-profit private laboratories and the manufacturers of diagnostic equipment.
- The most important contributions of a clinical microbiologist to the patient treatment process are participation in the identification of diagnostic procedures for the treatment of new disease and active consultation supported by the processing and analysis of sources of information on aetiological agents to meet the requirements of clinical practitioners. Such activities need to be placed on a strong conceptual foundation within the field with clear professional criteria so that clinical microbiology is not just laboratory work.

- The results of examinations in microbiology labs have long been recognised as a source of information. They have wide potential uses in surveillance and interdisciplinary cooperation. For this, a lab needs appropriate technical equipment, time and a set procedure for publishing reports in a permanent form that is broadly comparable and applicable.
- To find partners for cooperation or potential co-authors, new tasks or interesting problems to work on, and other ideas for improvement through work, it is necessary to create conditions for acquiring professional knowledge including study resources, consultation facilities, telephone lines, online access and study portals, to maintain international and domestic contacts and to free clinical microbiologists for consultation.

Automated serology for infectious diseases is a reality. Almost immediately after the identification of a new microorganism, techniques become available for detecting specific antigens and antibodies and new procedures are constantly being developed using more specific antigens, more detailed epitopes and antibodies to them. With so many new procedures, it is difficult to identify the "gold standard". Standards that lasted for years have been replaced by new laboratory procedures accompanied by new and more sophisticated diagnostic criteria. New enzyme analysis methods, nucleic acid amplification tests and the use of monoclonal antibodies are becoming accessible and usable in everyday practice. It is no problem to detect many different antigens, antibodies or the dynamic of their production. What is important is determining when an examination is indicated and how to interpret the results. A test for *clostridium difficile* bacteria using the PCR method will produce positive results for nearly everyone. What is the good of that? It is also important to ensure standardised conditions and laboratory quality to allow comparison of test results between laboratories. At present the causal identification of infectious diseases is based on the visualisation, cultivation and detection of antigens, nucleic acid or the metabolism and biochemical properties of the causative microorganism, or the detection of the corresponding classes of specific antibodies, the dynamic in the production of total antibodies against a specific agent or against certain structural or metabolic components. The test results require more than just confirmation of the responsible authorities for their application. There should be rules on the use of particular methods based on clinical correlation and the diagnostic value of the test. Every laboratory should determine the

quality of the test type and test kit. They should not rely on manufacturers' statements. A laboratory cannot keep its good name just by increasing the quantity of serum tested or introducing new test parameters. Quality can be maintained only through the professional ambition of well-educated, experienced and committed workers who are given room for professional growth. In a time when "cost-effectiveness" and "cost-benefits" are talked of at every level of healthcare organisations, qualified microbiologists and immunologists are in short supply. Hopefully they will not become unwanted. We need the machines to be good servants not bad masters. Both doctors and patients would benefit if the common examination and treatment units preserved the spirit of the clinical laboratory – a laboratory for the clinic – which provides a comprehensive service including the proposal of relevant examinations, the selection of the most appropriate test procedure with reproducible, precise and accurate results and the clearest possible interpretation. Each infectious disease has a range of investigation procedures, parameters and methods and the best combination to use can be accurately determined based on the infectious agent, the stage of illness, the experience of the treating physician, the options available in the laboratory and the interpretative value. It is not always possible to use just one method or just one principle to investigate many parameters. Turbidimetry is an accurate and excellent method, but it is not the most appropriate for detecting ASLO. Likewise, cytometric analysers are a great help in detecting significant bacteriuria by providing evidence for or against the presence of infection. There are still times when a urine culture examination is needed though, even in the case of a negative result. Just as clinical medicine cannot dispense with an individual approach and humanity, laboratory medicine cannot be left to machines and analysers.

8 LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	aquired immunodeficiency syndrom
ATB	antibiotic
CD	cluster of differentiation
CFR	complement fixation feaction
CNS	central nervous system
CRP	C reactive protein
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent analysis
ESDIS	European Surveillance of *Clostridium difficil*e Infections
FIA	fluorescence immuno assay
GLP	good laboratory practice
Hib	*Haemophilus influenz*ae type b
HPV	human papilloma virus
IDA	Immunodot analysis
IEM	immunoelectron microscopy
IEQASM	International External Quality Assessment Scheme for Microbiology
IFA	immunofluorescence assay
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IGRA	Interferon Gamma Release Assay
IL	Interleukin
IU/l	international units per litre
LCS	laboratory computer system
MAC	membrane attack complex
MALDI TOF	matrix-assisted laser desorption/ionization

MHC	major histocompatibility complex
ml	mililiter
MRSA	methicilin resistent *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards
NK	natural killer
NRC	National Reference Centre
OKM	clinical microbiology department (in Slovak: oddelenie klinickej mikrobiológie – OKM)
PCR	polymerase chain reaction
RF	rheumatoid factor
RIA	radio immuno assay
RIBA	recombinant Immunoblot assay
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SPIA	solid phase immuno assay
SVLZ	common examination and treatment units (in Slovak: spoločné vyšetrovacie a liečebné zložky – SVLZ
Th	T helper
ÚVZ	public health authority in Slovak - úrad verejného zdravotníctva
VNT	virus neutralisation test
WB	western blot
WHO	World Health Organisation

9 LIST OF FIGURES

10 CONTENTS PART 2

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TEXTBOOK REVIEW

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Textbook review:
Microbiology - principal and interpretation of serological tests

Authors: Doc. MUDr. Elena Nováková, PhD; Doc. MUDr. Jurina Sadloňová, PhD; MUDr. Martina Neuschlová, PhD; MUDr. Jana Kompaníková, PhD; MUDr. Vladimíra Sadloňová, PhD

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This 70-page text is intended to support supplementary study for students of general medicine and non-medical subjects at the Comenius University Jessenius Faculty of Medicine in Martin. It could also be used as a reference text for healthcare professionals.

It is mainly concerned with laboratory investigation methods in microbiology. The authors begin the work with a description of microbiology as a subject. They situate it in relation to other medical professions and subjects studied in medical faculties. They then discuss the basic principles governing the diagnosis of microbial diseases and explain the concepts of direct and indirect diagnosis and the elements of diagnostic procedures. An important part of the work is showing how laboratory results should be interpreted in unforeseen circumstances. The text also includes a list of abbreviations, a bibliography and useful pictures and diagrams. At the end of the work, the authors announce the work's planned second part, which will cover the use of the described procedures in the diagnosis of particular microbial diseases. The work is an updated

English version of the Slovak textbook *Mikrobiológia - princípy a interpretácia laboratórnych vyšetrení, I. časť*.

The textbook is a comprehensive work, describing in detail the most important aspects of immunoserological reactions. It has the potential to provide clear insights for students of microbiology, clinical microbiology and the laboratory diagnosis of diseases with microbial aetiology.

At Martin 14/12/2017

prof. MUDr. Mirko Zibolen, CSc.

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