MICROBIOLOGY - PRINCIPAL AND INTERPRETATION OF LABORATORY EXAMINATION

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

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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 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 to identify infectious agents based on a set of clinical signs indicative of a possible infectious origin (the microbial aetiology of a disease). 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.

The second completed edition of the textbook implements a specific chapter about the tests and interpretation of laboratory exam in some important clinical diseases. Our intention was to spread the knowledge for better understanding of the role of microbiology in the
diagnostical process. We are thankful for the support and help from cited authors, colleagues, students and our families. All materials are presented to be used for study purposes.
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 function 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 mucosa and helps to objectively determine and assess patients’ health condition. Its diagnostic activities make use of the possibility to establish direct proof of an aetiologic 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/).
MICROBIOLOGY AS A SCIENCE

Microbiology (from the Greek μικρος, mikros, “small”; βιος, 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 subdisciplines 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:
  o the taxonomy of microorganisms: the study of the nomenclature and classification of microorganisms,
  o 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:
  o microbiology of plants: study of the interaction of microorganisms, plant pathogens and plants,
  o soil microbiology: the study of microorganisms 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).
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 to perform follow-up 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” leading to 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 every country. 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 comprehensive microbiological examination that makes a significant contribution to the individual patient’s treatment and care by establishing an aetiological diagnosis need not require a very extensive range of diagnostic instruments. The ability of a microbiological laboratory to provide adequate diagnoses 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 diagnostic methods are usually dependent on a microorganism’s ability to grow, which means that 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 has been 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 interference with 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 of a non-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 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 local resistance levels. 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 this class of medicines. Microbiological examination is an important tool for its development. Rules governing antibiotic use should ensure the required efficacy and safety of antimicrobial therapy, limit the emergence and spread of resistant microorganisms and 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 cultivated 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. They can respond to 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 titre. The titre 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 infections.

The aggregate results of serological examinations – immunological surveys – are used to 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 the population 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 to a chosen disease. They are the basis of the 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 – KFR or VNT for poliomyelitis, IgG antibodies against diphtheria or antitoxic antibodies against the toxin *Corynebacterium diphtheriae*),
– standard conditions in 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 of 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 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 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, immunocompromised individuals, non-responders). Such people are at risk of acute infection and potential susceptible individuals, even though the occurrence of disease is epidemiologically unlikely. It is possible for an aetiological agent to be introduced from a country where collective immunity is below the threshold for preventing 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 be based on 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 positive and negative results. The boundary value of an antibody detection 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 inadvisable, because it increases the level of uncertainty in interpretation. Serological tests have certain objective and subjective limitations that 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 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 malignant disease development if a person is already infected. 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 lead to a 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 a full version the aim is to identify all cases of disease occurrence (as in the nationwide surveillance of invasive Hib infections in Slovakia). A “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 for the human eye 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 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 limit 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 cause 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 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 promoting rational use of antibiotics in both outpatient and hospital practice.
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 development 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) 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 (Bhattacharya, 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 the 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 linking 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 the 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, 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 on top of 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) 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 ways microorganisms function 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 pharmaceuticals. 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 and 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 testing for antibodies. A laboratory is able to detect a parasite from biological material during the prepatent period after infection. A doctor need not 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, meaning that they must maintain both theoretical readiness and the practical resources necessary for timely diagnosis. Newly emerging infections are often acute and can create a panic. In recent years increased numbers of foreign nationals have been coming to work in Slovakia.

Microbiological laboratories are asked to identify aetiological agents in samples from a wider region and the potential number is very large. The information needed to manage a 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 for 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, how contaminated materials and instruments and their handling contribute 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.
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 immunoserosology 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 is worth the investigation and the wait for results for its contribution to informed decision-making. 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 the care of top professionals and Michelin-star levels of service.

This textbook aims to provide a general description 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. A second part currently in preparation 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). Specific 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 or 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 by which this specific hypersensitivity can be detected. 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 is 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 do not usually 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 a 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 aggregation-promoting conformational changes in the viruses’ structure. 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 when it is released into the blood from the target tissue where it proliferates (secondary viremia). There are 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 known as the 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 bound with MHC-I molecules and presented on the infected cell’s surface, 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 and 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 based 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 an 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. The foundation of laboratory procedures using serological reactions or immunodiagnostic approaches is the reaction between an antigen and a specific antibody in a certain medium.

The components of a serological reaction can have different characteristics, which determine the methods that should be used:

- **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 procedures, 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.

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.
Recombinant antigens – some microorganisms, such as *E. coli* bacteria, are used in the preparation of human or other antigens. The technique exploits bacterial cells’ capability for heterologous gene expression. When genetic information for a required antigen is correctly added to the *E. coli* genome, the foreign genetic material is transcribed and translated to produce the antigen. Part of the process involves purifying the desired antigen from *E. coli*’s own antigens, which can otherwise 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.

**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 their 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.
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 microtitre 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 microtitre 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.
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 detection of 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 its 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 2 samples taken over 14–21 days. Different stages of disease are characterised by the presence of specific antibodies of different isotypes.

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.
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 produced during acute infection but persist for 3–6 months after primary infection. In a newborn infant, it is a sign of intrauterine infection. Because the presence of IgM in serum is temporary, a finding indicates a recent acute infection and it can usually be confirmed with one sample. Nevertheless, 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 a 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**

<table>
<thead>
<tr>
<th>Stage of infection</th>
<th>IgM</th>
<th>IgA</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute infection</td>
<td>+</td>
<td>- (+)</td>
<td>-</td>
</tr>
<tr>
<td>Post-acute phase, convalescence</td>
<td>+</td>
<td>+ (-)</td>
<td>+</td>
</tr>
<tr>
<td>Anamnestic antibodies, overcome infection, post-vaccination antibodies</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Re-exposure to causal agent</td>
<td>Re-activation of chronic infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td></td>
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<tr>
<td>+</td>
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<td></td>
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</tr>
</tbody>
</table>

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

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).
6.3.1 Antigen detection

SPIA – solid phase immunoassay – 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.

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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.

**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 the solid 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).
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** – 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 or of antigens dissolved 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 not virus antigens, which are smaller and require a larger antigenic mass to create visible clumps.

**Immunofluorescence uses a fluorescent dye that binds covalently with 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.**

**Latex agglutination** – latex-bound specific antibodies capture microorganisms in the sample.
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 and 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 labelled detection
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 adsorbed to 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.
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 involving radionuclides is no longer 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. An antigen is added to the serum 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 systematise 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 inverse of the serum dilution (titre 128).
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.

6.4 Immunoserological methods for the diagnosis of infectious diseases

6.4.1 Non-specific indicators of infectious diseases

Acute inflammation is produced by the local response to infection, tissue damage or both. Its key features are vascular changes and the accumulation of leukocytes. The systemic and metabolic changes that take place in the first days after infection constitute the acute phase inflammatory response. Acute phase proteins are those whose concentration increases sharply during this phase. The concentration of complement factors and ferritin increase by nearly half, there is a more than 200% increase in the concentration of alpha 1-antitrypsin, fibrinogen and haptoglobin and a nearly thousand-fold increase in the concentration of C-reactive protein in response to bacterial and fungal infections or severe tissue damage. CRP and complement
factors are the only proteins in the acute phase inflammatory response that have been demonstrated to be effective in the physical elimination of microorganisms.

**CRP**

C-reactive protein is an important marker of the acute phase of inflammation. It is a protein produced in the liver and present in serum in minimal concentrations under baseline physiological conditions. It acquired its name because of its ability to react with the C-substance in the capsule of the gram-positive pneumococcus bacterium - *Streptococcus pneumoniae*. It is not, however, a specific anti-pneumococcal protein. Its increased synthesis is triggered in the acute phase of any bacterial or fungal inflammation and after severe tissue destruction. CRP is able to bind to phosphocholine and galactose residues, which are binding sites present on the microbial products of bacteria, moulds and some parasites. After binding to these surfaces, it activates the complement system via C1q, which increases opsonisation. In mouse experiments, human CRP provides in vivo protection against lethal doses of *Str. pneumoniae*. Its concentration increases from its normal range to hundreds of micrograms per millilitre in 3 days. The highest levels are in patients with bacterial infections (over 100 micrograms per millilitre). Chronic inflammation, autoimmune diseases, cancer, toxic hepatitis, heart failure and pregnancy are associated with moderate increases in its concentration. Monitoring of CRP dynamics in chronic inflammation can confirm a bacterial or fungal superinfection. The dynamics of CRP concentration are important for monitoring disease status. CRP rises faster and decreases earlier during recovery from an infection compared to the erythrocyte sedimentation rate and leukocyte count. CRP can also be measured in other body fluids. The presence of CRP in cerebrospinal fluid is a sensitive and specific method in the differentiation of viral and bacterial infections of the brain. CRP testing is also justified for distinguishing patients with bacteraemia from patients with a positive blood culture result caused by contamination, for monitoring paraplegic patients for early diagnosis of urinary tract infections, for distinguishing between cystitis and pyelonephritis in children, for distinguishing between bacterial and viral pneumonia in children and adults and for distinguishing between bacterial pneumonia and acute bronchitis.

**Endotoxin**

Endotoxins are lipopolysaccharides (LPS), molecules found in the outer membrane of gram-negative bacteria. In vivo, it has toxic, pyrogenic and other biological effects resulting from the release of cytokines (interleukin-1, tumour necrosis factor), which have beneficial effects
for the non-specific immune response at low concentrations. Higher concentrations of endotoxin can cause metabolic and pathophysiological cascades leading to sepsis or gram-negative endotoxin shock. If defence measures are successful, endotoxin is phagocyted and detoxified in the liver. During infection, LPS concentration is detectable only in the portal vein and not in peripheral circulation. Endotoxin is detected using the limulus amoebocyte lysate (LAL) test. It detects endotoxin traces based on the ability of LPS to coagulate the blood of the crab *Limulus polyphemus*. This test is not for a specific microorganism but detects the presence of endotoxin, which is a part of the cell membrane of gram-negative bacteria. Endotoxin is very quickly enveloped by phagocytes and its detection in peripheral blood is not clinically significant. On the other hand, if found in CSF, it is a sensitive indicator of gram-negative bacterial meningitis.

**TNF**

Tumour necrosis factor – cachexin is a cytokine that participates in the immune response against microorganisms. It also influences anti-tumour responses, which is how it got its name. Experiments on mice with a tumour present found that tumour necrosis and regression followed the injection of serum containing TNF. This factor was also identified in the search for the substance responsible for cachexia – wasting syndrome – associated with parasitic diseases. TNF and cachexin were originally thought to be separate substances but DNA sequencing showed that they were homologous, and they are now considered to be identical. In experiments on laboratory mice, TNF induces metabolic acidosis, haemoconcentration, changes in glucose metabolism, pulmonary oedema, haemorrhagic adrenal and pancreatic necrosis and tubular necrosis of the kidneys. After TNF neutralisation combined with administration of specific antibodies, these symptoms can be overcome. At present anti-TNF antibodies are used for clearly defined indications in the “biological treatment” of some autoimmune or chronic inflammatory diseases (Crohn’s disease, progressive polyarthritis). It is possible to determine concentrations of TNF in human serum. There is information on the correlation between TNF levels and the degree of septic shock and risk of death for patients with meningococcaemia. Although TNF is a non-specific marker, a non-specifically induced cytotoxin, its detection can provide information on the extent or character of damage in some infection processes.
6.4.2 Antibody types and their use in diagnosis

Polyclonal antibodies

The basic components of immunoserological reactions used in immunoassays for infectious diseases are antibodies, antigens and the medium in which the reaction takes place. An immunoserological reaction is always a reaction between an antigen and an antibody. Their properties and characteristics determine the selection of the laboratory method and the possibilities for visualising the antigen-antibody complex. Reactions can be used to identify unknown antibodies using a known antigen or an unknown antigen using known antibodies. The choice of method depends on the physical properties of the antigen, its solubility (antigens are classified as soluble or corpuscular). The quality of antibodies is related to high specificity. However, non-specific and cross-reactive antibodies also have their uses, such as in the VDRL test used to screen for syphilis.

The antisera used in immunoserological assays are prepared by the hyperimmunisation of animals using purified antigens with an adjuvant. The sera of immunised animals are tested for monospecificity. If contaminating antibodies are present, or when using non-specific sera, undesired antibodies are removed by adsorption using the corresponding antigen. The immunoglobulin fraction is obtained from serum by selective precipitation with ammonium sulphate or by chromatography. Even in hyperimmune sera, specific antibodies make up only 10% to 15% of the total IgG fraction of polyclonal antibodies. Other IgG molecules are of unknown specificity reflecting the mixture of antigens that the animal has been exposed to during its life. Much work is often needed to identify all the polyclonal antibodies in order to detect only those that are desired. The other 85% to 90% increase the incidence of non-specific binding of conjugated (but unwanted) antibodies in the test system.

Purified antibodies

To eliminate antibodies with unwanted specificity, the IgG fraction of polyclonal antibodies can be purified by binding them to an insoluble antigen and flushing. This can be achieved using a form of column chromatography.

The immunising antigen is bound to a matrix together with an inert molecule performing the function of a spacer. The spacer is covalently bound to both the antigen and the solid phase in a way that preserves the important antigenic determinants. Antibodies are then added to the column, which maintains optimal conditions for the reaction of the antigen and antibody (pH 7.5 to 8.5; normal physiological ion composition of the solution). IgG molecules of non-matching specificity are removed by flushing with a wash buffer. The antigen-specific IgG
molecules are removed from the antigen matrix by flushing with an elution buffer with a lower pH and a lower ion concentration. This preserves the functional integrity of the IgG molecules while gently detaching the antibodies from the insoluble antigen. Antibodies obtained by this method have significantly higher specificity (85% to 90%). This procedure reduces or entirely eliminates non-specific binding with other antibodies, which ensures greater specificity in immunological assays. Polyclonal antibodies that have been purified and thus have greater affinity can be used for various purposes. Most of the antibodies against human immunoglobulin conjugated with a fluorescent dye or an enzyme that are used in available laboratory assays are purified polyclonal antibodies with high affinity. Polyclonal antibodies bind to multiple antigenic determinants, which increases their ability to detect the corresponding antigens.

**Fractionated antibodies**

Some antibodies in sera used for antigen detection bind non-specifically to IgG Fc receptor cells. An effective way to reduce such non-specific binding is to fractionate the IgG antiserum to eliminate the Fc region of the IgG molecule. In controlled conditions, the pepsin enzyme separates the C-terminus of the IgG molecule (the Fc fragment). The result is an N-terminal fragment binding the F(ab)2 region. These F(ab)2 fragments can be conjugated with fluorochrome or enzymes. Purified polyclonal antibodies with increased affinity can be treated with pepsin to create affinity-purified F(ab)2 antisera.

**Monoclonal antibodies**

The production of monoclonal antibodies was first described by Kohler and Milstein in 1975. They were immediately found to have immense potential for use in clinical laboratory tests and immunotherapy. Monoclonal antibodies (MAb) are prepared by hybridising an antibody-producing cell (B lymphocyte, plasma cell) with a replicating cell line. Any antibody-producing cell correctly programmed to synthesise an antibody of a single specificity with one type of heavy and one light chain type can clone and replicate them almost indefinitely. These antibody-producing cell clones (hybridomas) are used to produce an almost unlimited quantity of monoclonal antibodies with defined properties. Hybridomas can be stored for a long time in liquid nitrogen. When needed, they can be regenerated in the form of tissue cultures or applied to laboratory mice, in which they produce tumour ascites, from which the monoclonal antibodies can be isolated. Monoclonal antibodies have clear advantages. They require only minimal purification and only one test of specificity. Monoclonal antibodies do not usually
bind non-specifically with human IgG Fc fragments and do not usually produce cross-reactions and non-specific reactions. Their disadvantage is that they cannot be used in assays where cross-binding of antigen and antibody is required, such as agglutination and precipitation assays, although mixtures (cocktails) of multiple monoclonal antibodies may partially solve this problem. Monoclonal antibodies produced in mice also fail to bind to the C1q complement component, meaning that they do not activate the complement cascade. Furthermore, the single antigen determinant against which the monoclonal antibody is directed need not be expressed in some cases of antigen presentation (live and fixed microorganisms). Provided that their potential and limitations are taken into account, monoclonal antibodies have almost unlimited use in treatment and diagnosis.

6.4.3 Antigens
6.4.3.1 Soluble antigens and their reactions with antibodies

Double diffusion in agar (Ouchterlony reaction)

This classical method of antibody detection permits an evaluation of their specificity (identity, partial identity and non-identity – see the illustration above). It can also be used in the characterisation of antigens if antibodies with known specificity are available. The method requires 18-24 hours of diffusion and cannot be used for the rapid diagnosis of acute illnesses. It retains valid uses for the detection of precipitating antibodies in suspected histoplasmosis, coccidioidomycosis or aspergillosis or other fungal antigens associated with hypersensitivity pneumonitis. A modified form, Elek’s test, is used to identify toxigenic strains of *Corynebacterium diphtheriae*.

Immunodiffusion in agar

Under standardised conditions, this is an accurate and useful method of visualising antigen-antibody reactions in agar. After fixation and staining, the results can be archived and documented. The method can produce quantified results and it was one of the first methods in which computers were used to calculate concentrations and variations for a set of tests. Although, like Ouchterlony diffusion, the method is not a rapid diagnostic test, it can be used to identify almost any antibody or antigen. One advantage is the ability to visualise an absolute zero value (IgA deficit), where automatic computer-controlled systems cannot guarantee results below a minimum concentration near zero. This makes it useful for distinguishing reduced IgA antibody production from an inability to produce antibodies, although other methods can also be used for this purpose (e.g. ELISA, immunoassay). Given
the relative infrequency of such a disability in the population, waiting for a sufficient number of patients to justify use of the test could take several months. Nevertheless, the quality of the test and its affordability justify keeping it in service. With this type of test, an experienced diagnostician can detect a non-normal image of diffused antibodies and raise the alarm if there are interfering, non-specific or pathological antibodies.

**Counterimmunoelectrophoresis (CIE)**
This is one-dimensional double electrophoresis which specifically directs the movement of antigens and antibodies against each other in an electric field. The pH environment is set up to direct antibodies towards the cathode (negatively charged electrode) and antigens to the anode (positively charged electrode). This movement rapidly concentrates antigens and antibodies in the area between the starting wells. The method is more sensitive than double diffusion. It was first used for the detection of HBsAg. It is useful for the rapid diagnosis/identification of antigens from bacteria that cause some serious infections (meningitis, septicaemia, DIC, pneumonia). Although it has been largely replaced by less laborious latex agglutination methods, it is useful for proving antigens for which commercial sets are not available, and therefore also for experimental purposes. Depending on the quality (sensitivity and specificity) of the antibodies used, the minimum detectable concentration of a bacterial antigen under this method is 50 to 10 ng/ml.

6.4.3.2 Corpuscular antigens and their reactions with antibodies

**Hemagglutination**
Hemagglutination is a method for making visible antigen-antibody reactions that is both sensitive and specific. A quantity of antigens can be bound to erythrocytes, which become an indicator system for detecting antigen-antibody binding. Carbohydrate antigens are able to bind to erythrocytes directly. Protein antigens need to be treated with tannin. Tannin-treated erythrocytes increased the sensitivity of the test. The tannin-treated erythrocytes bound with protein or carbohydrate antigens can be stored permanently if they receive follow-up treatment with formalin or glutaraldehyde. The assay was used to detect antibodies against the toxins *Corynebacterium diphtheria* and *Clostridium tetanus*. *Treponema pallidum* antigens absorbed into tannin-treated erythrocytes are used to detect specific antibodies in the MHA-TP micro-emulsion assay for *T. pallidum*
**Hemagglutination inhibition test – HIT**

The principle of this test is based on the ability of various virus antigens (hemagglutinins) to agglutinate the red blood cells of certain animal or bird species. Specific antibodies against these antigens in human serum inhibit such spontaneous agglutination so the inhibition the hemagglutination of red blood cells indicates the presence of specific antibodies against virus antigens. This test was originally used to detect antibodies against the rubella virus. A disadvantage is its inability to distinguish between IgG and IgM antibodies. It is also used to detect antibodies against some other viruses – influenza viruses, arboviruses, reoviruses and some enteroviruses.

**Direct agglutination** – A traditional method using specific antibodies in a solution. 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 agents of meningitis in CSF or when there are 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 not virus antigens, which are smaller and require a larger antigenic mass to create visible clumps.

**Latex agglutination**

Latex particles are polystyrene beads that bind to the Fc fragment of an IgG molecule. The unoccupied Fab regions of the antibody molecule can bind with a specific antigen. Target antigens with recurring antigenic determinants (polysaccharides), multivalent antibodies bound to multiple latex particles bind to antigenic molecules and link the latex particles to each other, producing visible agglutination. The latex particles function as an indicator system to visualise the antigen-antibody reaction. In theory, every antigen (but not every hapten) against which antibodies are produced can be detected by latex agglutination. At present it is used to detect free polysaccharide antigens in CSF and urine. Latex agglutination has replaced CIE for the detection of polysaccharide antigens in CSF (N. meningitidis, H. influenzae b, S. pneumoniae, E. coli, S. agalactiae) or the moulds Cryptococcus neoformans and Candida albicans.

**Coagglutination**

*Staphylococcus aureus* has protein A on its cell wall surface. This protein is able to bind to Fc fragments of IgG subclasses 1, 2 and 4 in the same way that Fc fragments bind to latex
particles. staphylococcus bacteria covered with antibodies can thus be used as an indicator for the detection of an antigen, the antibody against which is non-specifically bound to its surface. Coagglutination was used to detect bacterial antigens in CSF. It is used in the immunological typing of streptococcus bacteria and for detecting the production of *staph. aureus* coagulase. any cluster of antigen and the corresponding IgG can bind to protein A via their Fc fragments, which frequently results in non-specific reactions. Non-specific reactions can be avoided by treating the bodily fluid to be tested with soluble protein A to block the binding of immune complexes. Another option is to heat the bodily fluid to 100°C, which denatures the patient’s IgG molecules before testing. Coagglutination is very sensitive to non-specific reactions; a disadvantage is its short duration due to the risk of denaturing the bacteria. Most of the tests where co-agglutination was used have been replaced by latex agglutination or immunoassay.

6.4.4 Lytic reactions

**Complement fixation reaction**

One of the most widely used of serological tests was this two-stage procedure that exploits the lysis reaction between complement proteins and indicator red blood cells. The first stage in the procedure is the reaction of antigen and antibody in the presence of complement proteins. The complement proteins used in the test must be standardised for lytic activity. Titration of commercial animal complement proteins is carried out before testing to determine their lytic activity. To ensure that the system does not contain two types of complement proteins (the standardised ones and the natural ones in the test serum), the sample is heated to 56°C before testing to destroy its complement proteins. If a specific antibody is present in the test serum, it will create a complex with an added antigen which can activate the complement cascade via classical pathways. This uses up the complement in the test system.

In the second stage, animal red blood cells combined with anti-erythrocyte antibodies are added to the first stage as an indicator system.

If specific antibodies were present in the first step of the test, they would have bound to the antigen and thereby activated the complement cascade. This would have used up the complement proteins and none would be left to cause lysis of the red blood cells in the presence of their antibodies during the second stage of the reaction.
A positive result (presence of antibodies in the patient’s serum) is indicated by the absence of haemolysis.

A different outcome will be observed if specific antibodies against the antigen are not present in the first stage reaction. In this case no Ag-Ab immune complexes are created; the complement cascade is not initiated; and the complement proteins are not consumed. They remain available and after the addition of the indicator system (red blood cells and antibodies against them), the complement cascade is activated, and haemolysis takes place. Haemolysis indicates a negative result. CFR is a semi-quantitative method that can be used to detect an antigen or antibody depending on how the test is set up. CFR is unable to distinguish IgG from IgM because both classes are able to bind with complement proteins. It is exceptionally sensitive and very specific for qualitative diagnosis (especially for antigens). Despite producing good results, its demanding requirements meant that it was only used in laboratories with a good laboratory tradition and robust quality standards. It is a method for testing for antibodies against less prevalent infections. One advantage is that apart from the specific antigen, all the components of the test system can be used without change in multiple tests.

Agglutination and lytic tests can be set up to produce qualitative results or quantitative results by specification of the titre. The titre is the inverse of the serum dilution in the last sample that gives a positive reaction. The test must be set up so that the quantity of antibody in the serum is equal to the quantity of antigen used, and the reaction must be in the zone of equivalence. An excess of antigen or antibody can lead to a false negative result.

Some bacteria, such as *Streptococcus agalactiae* – beta-haemolytic group B streptococcus – produce a variety of extracellular toxins that can stimulate antibody production in infected patients. Some of these toxins can act as haemolysins and specific antibodies neutralise their toxic function and prevent haemolysis. As with the CFR, the absence of haemolysis indicates a positive result. A negative result – the absence of specific antibodies - is indicated by the lysis of the red blood cells. The most frequently used test is the ASLO titration test, which provides immunological evidence of an invasive *Str. pyogenes* infection.
6.4.5 Immunofluorescence assays

Immunofluorescence assays are the most commonly used immunohistochemical technique. Immunohistochemistry is an approach in which a sample of tissue or cells (often taken from a monkey’s oesophagus or kidneys, amongst other sources) is used to capture specific antibodies from the patient's serum (immunoglobulins) against a target antigen. The resulting complex is then identified by a conjugate with a chemical marker (e.g. a fluorescent dye such as fluorochrome). In immunofluorescence, the tissue or cell sample is the substrate (the source of the antigen) attached to a slide and the fluorochrome-conjugated antibody is a detection system. Immunofluorescence remains the “gold standard” for the serological diagnosis of many infectious diseases. One benefit of immunofluorescence is that the substrate can be visualized, which ensures specificity for certain reactions. Another is that the method is significantly less laborious than some other methods, notably CFR. It is also highly reproducible in routine work and, in indirect immunofluorescence, the same conjugate can be used to detect antibodies against multiple antigens. This means that it is possible for a single sample to be screened for multiple antibodies or antigens on a single slide in differential diagnosis, a procedure that can lead to a significantly quicker diagnosis. A disadvantage of
immunofluorescence is that it requires a fresh or frozen tissue or cell sample and that tissues or coatings used for Gram staining cannot be used. Furthermore, the method requires special equipment – a fluorescence microscope – and intensive laboratory work in multiple steps including dilution, multiple incubations, washing and cover slides. Finally, the count is subjective and therefore requires experience and multiple controls for specificity and sensitivity. The method’s speed and potential for individualisation make it particularly suitable for experimental work.

**Direct immunofluorescence**

Direct fluorescence is used to detect antigens or organisms present in cells or tissues using a fluorochrome-tagged antiserum (conjugate) specific to the target antigen. Direct immunofluorescence is an important means for detecting the presence of antigens in tissue (elementary bodies of *Chlamydia trachomatis* in genital epithelial cells, *Treponema pallidum* from the site of ulceration or mucocutaneous lesion, *Borrelia burgdorferi* from the site of infection and the identification in tissue of microorganisms that are difficult or impossible to cultivate – the HSV, CMV, RSV and VZV viruses, *Legionella pneumophila, Leptosira sp.*).

![Direct immunofluorescence](image)

**Indirect immunofluorescence**

Indirect immunofluorescence is used mainly to detect antibodies in a patient’s serum. A known antigen is fixed to a slide. The patient’s serum applied to the appropriate substrate site is incubated to create an antigen-antibody complex. Unbound, non-specific antibodies are removed by washing. The bound, specific antibodies are then incubated with a fluorescent conjugate that binds with the existing antigen-antibody complex (a fluorescent conjugate of an antibody against the immunocomplex formed by the antigen + serum specific antibodies) The fluorescent conjugate is thus the third layer on the slide. Indirect immunofluorescence can be used to detect IgG, Ig total or IgM antibodies.
The antibodies against human immunoglobulin that are typically conjugated with fluorescein are those against IgG against kappa and lambda light chains. The reactivity with light chains can also detect IgA and IgM, which may result in the test detecting Ig total. If the test is designed to identify antibodies during an acute infection, the conjugate must be specific for IgM heavy chains and not light chains or heavy chains with different specificity.

Immunofluorescence is part of the classic test for antibodies against Treponema pallidum, the fluorescent treponemal antibody absorption (FTA-ABS) test. An antigen from the Nichols strain of Treponema pallidum is fixed on a slide. Before incubation the patient’s serum is incubated with the non-pathogenic Reiter strain of Treponema pallidum. This procedure significantly increases the test’s sensitivity. False positive reactions can be obtained if patients have autoimmune reactions, hypergammaglobulinemia and borreliosis because of the cross-reactivity of antigenic epitopes common to Spirochetes. At present, fluorescence plays an important role in the detection of antinuclear antibodies and other types of antibodies related to autoimmune diseases.

Amplification – increasing sensitivity

When using immunofluorescence methods, sensitivity is limited by the need for the intensity of the fluorescence to be visible to the human eye. The optimal ratio of fluorescein and protein in the conjugate is 2.5. This means 2-3 molecules of fluorescein per immunoglobulin molecule, which is achievable in the case of bacterial or parasitic surface antigens. Directly conjugating antibodies with a higher ratio of fluorescein and protein can increase non-specific staining. When testing for low-density antigens or using monoclonal antibodies, it is sometimes necessary to use a higher ratio of fluorescein and protein to achieve sufficient sensitivity. Indirect immunofluorescence or double indirect immunofluorescence are used to amplify the fluorescence signal and improve sensitivity.

If antigens can be detected by direct fluorescence, indirect fluorescence increases the sensitivity of the test. If it is difficult to detect antibodies using indirect fluorescence, double indirect fluorescence will improve detection by increasing the sensitivity of the assay. If an
organism is not visible when using direct immunofluorescence, the test can be repeated with unconjugated antibodies (e.g. from rabbits). Washing can remove all the unbound antibodies and the bound rabbit antibodies can be detected using fluorescein conjugated with goat anti-rabbit IgG antibodies. Every rabbit IgG molecule has many antigenic sites that will be recognised by the goat anti-rabbit IgG antibodies. If four goat molecules against rabbit IgG molecules bind to rabbit IgG, the intensity of fluorescence will increase fourfold. Any additional step to increase sensitivity requires additional controls to ensure specificity is not impaired.

Complement-amplified fluorescence also known as anti-complement fluorescence was used to detect the herpes virus. Tissue infected with herpesviruses (cells from tissue cultures) have increased expression of the Fc receptors for IgG, which non-specifically bind IgG antibody molecules. Anti-complement indirect fluorescence uses complement as the third of four layers. The antigen is the substrate on the slide, the patient’s serum is added in the same way as in indirect immunofluorescence. A source of active (guinea pig) complement is added in the next step. IgG molecules bound to the Fc receptors cannot bind C1q. IgG or IgM bound to the antigen by the Fab fragment does not bind C1q and activates the classical complement cascade. After removal of the unbound complement and other proteins by washing, a Fab2 fluorescent conjugate that binds to guinea-pig C3 is applied. The anti-complement indirect immunofluorescence eliminates the need to check for non-specific IgG binding because the complement component is detected instead of IgG. This type of test is used as a diagnosis for antibodies against certain herpes antigens (EBNA EBV).

Avidin–Biotin Complex is a very effective amplification method. Biotin binds covalently with antibodies and conjugated avidin provides an additional level. Avidin has a strong binding affinity for biotin and every molecule of avidin can bind four biotin molecules. Avidin can be conjugated with fluorescein molecules without loss of its ability to bind to biotin. This means that it achieves very clear specific staining with minimal non-specific reactions. Conjugates of avidin and non-specific antibodies bound to biotin are used as controls. An advantage of the avidin-biotin complex is that the same biotin-bound antibody can used with multiple differently conjugated avidins e.g. fluorescein (green fluorescence), phycoerythrin (red fluorescence), peroxidase (light microscopy), ferritin (electron microscopy). Avidin biotin complex is useful for working with monoclonal antibodies
because binding to biotin is fast, does not require intensive laboratory work, and keeps the same ability to bind the antigen.

6.4.6 Immunoenzyme techniques

Rapid screen tests

Rapid diagnostic tests for the presence of an antigen or antibodies in a clinical sample are of interest to first contact physicians because they can reduce the time for diagnostic decisions and allow causal therapy to be applied or allow a suspicion to be eliminated without a long wait. The basic qualitative requirements are the technical parameters and handling requirements (a simple procedure, inexpensive equipment, a short interval until the results are available – at most 10-15 minutes). Colloidal gold labelled antibodies are used in the diagnosis of the antigen, which is based on the capillary flow of the antigen against the detection antibodies. These are immobilised on a paper strip and migrate after the strip is wet with the sample. The gold-labelled indicator agent (made up of anti-human immunoglobulins) is fixed horizontally on the strip (-). A vertical line is created if the colloid gold labelled solution interacts with the immobilised anti-human antibodies. Rapid screen tests for antibody detection are more sensitive than tests for antigen detection. They achieve sensitivity close to classical enzyme immunoassays.

Rapid enzyme immunoassay for antigen detection (EIA) is a method that has been found in recent years not so much in clinical laboratories as in first-line offices, small non-specialised laboratories intended to provide rapid and automated diagnosis, and in some cases among the general population. EIA is used in techniques detecting protein antigens. The method usually
indicates the presence or absence of antigens by means of colour changes. Most such methods have been developed for rapid screening. They are very simple and do not require special technical or laboratory equipment or trained personnel. They can be done in a doctor’s office. Despite their high price they benefit both doctors and patients. Confirmation of streptococcal angina means that causal treatment with penicillin can be applied and broad-spectrum antibiotics do not need to be used so frequently. Clinical laboratories do not use these tests, partly because of their high price but mainly because they do not provide adequate sensitivity or specificity when precise identification is important or follow-up tests are required, for example to determine susceptibility. The latest techniques use liposomes, artificial spherical lipid particles, to detect some antigens (group A streptococci). In this test a specific antibody is adsorbed on porous material. The patient’s sample is applied to the surface and any antigen that it contains is bound to the antibody. Liposomes covered with antibodies against the same antigen and containing pigment inside the concentric layers are then passed through the site of the ongoing test. If no antigen from the sample has bound to the antibody in the porous material, the liposomes pass through the porous material. If the liposomes are bound, the pigment is released by a washing solution that breaks up the liposomes and deposits the pigment in the matrix.

The incorrect taking of samples can be a problem for such procedures. For example, in the test to prove the antigen *Chlamydia trachomatis*, a negative result could mean “antigen not present” or “incorrectly taken sample”. Rapid EIA tests for sexually transmitted diseases can be made available to outpatient doctors and the public – like baby tests. On the other hand, these tests can provide no additional information such as information on ATB susceptibility, nor do they contribute to epidemiological research. In view of these problems, clinical microbiologists and everyone who uses such tests should be made aware of their limitations.

**ELISA – enzyme-linked immunosorbent assay**

ELISA methods are the gold standard in the diagnosis of infectious diseases and their variability and the simplicity of preparing homemade sets make them suitable for the research and development of new diagnostic tools. Enzyme-linked immunoassays were first described by Engvall and Perlman in 1971. They used Nakan and Pierce’s method to conjugate horseradish peroxidase enzyme with immunoglobulin and used this conjugate to detect specific immunoglobulin on a microtitre plate. They called the method ELISA - enzyme linked immunosorbent analysis. Initially, ELISA was less sensitive than RIA, but as both methods were enhanced with additional steps that increased their sensitivity and introduced
new ways of conjugating enzymes, the two methods achieved professional parity. The wider spread of the ELISA method is due to its lower price and lower safety costs. An antigen for antibody detection is fixed to a solid base (polyethylene well). To it is added a sample of the patient’s serum to be tested for the specific antibody. If it is present, it will bind to the antigen adsorbed on the solid phase. Follow-up washing removes the unbound serum with non-specific antibodies. A conjugate is then applied (an antibody to the bound complex – anti human enzyme conjugated antibody – an antibody against human immunoglobulin conjugated with an enzyme) Washing removes the unbound conjugate. A substrate is then applied that breaks down the present enzyme conjugate, resulting in a colour change. The intensity of the colour change can be measured photometrically and permits the test to be used for quantitative purposes.

**Technical components of the ELISA method**

**Solid phase**

ELISA is the most widespread solid-phase enzyme immunoassay. Polyethylene, PVC, nitrocellulose, agarose, glass, cellulose, polyacrylic and dextran surfaces can be used. The classic 96 well plastic plate is perhaps the most widespread solid phase used for enzyme immunoassay. Today tests are also conducted using 8-well strips or individual wells to test smaller numbers of samples. Plastic balls have also been adopted by certain companies as a closed diagnostic system (an analyser for just one type of diagnosis). A known antibody is adsorbed to this surface either passively or using covalent bonding when testing for an antigen; a known antigen is adsorbed when testing for an antibody. Laboratories can prepare their own (homemade) diagnostic plates with wells covered with specific antigens. The methods do not require special equipment, but it can be difficult to achieve accuracy and standardisation or to find the best concentration of the components. The most important step is the precise and careful washing of all the wells on the plate to remove residual reagents that could block subsequent stages or cause a false positive result. ELISA became accessible for routine work when microtitre plates became commercially available in sets that could provide all the diagnoses for a complete test.

**Conjugate**

In the next step the conjugate is introduced. It comprises an enzyme bound with an antigen or antibody depending on the type of enzyme immunoassay. Commonly used enzymes include horseradish peroxidase and alkaline phosphatase amongst others. The most important properties affecting the selection of the enzyme for the conjugate are stability, cost
affordability, simple conjugation, non-presence in human serum and easy detection. The enzyme can be conjugated with various uptake molecules (polyclonal antibodies, IgG fractions, polyclonal antibody Fab fragments, purified antibodies, monoclonal antibodies). A homemade test requires a compromise between optimal results and the lowest price. Polyclonal antibodies are usually the most affordable, but they are not purified and carry the risk of a non-specific reaction. The best results come from purified affinity antibodies, which can also be obtained as commercial preparations. Horseradish peroxidase conjugates are cheaper. The most expensive but also the most specific conjugates use monoclonal antibodies. For a homemade technique, it is important to determine the appropriate dilution, meaning that the absorbance of the positive assay is greater than 1.0. The range of conjugates available is highly diverse. Nevertheless, it can sometimes be necessary to create a conjugate in the laboratory for special purposes. Before conjugation, it is important to achieve an appropriate concentration of antibodies and to remove other proteins so that they are not marked by the enzyme. This can be done using ion exchange, chromatography or ammonium sulphate. The next step is the conjugation itself. A buffer containing bovine serum albumin or gelatine is used to block sites that were not conjugated or to saturate less than optimally coated well surfaces. To prevent possible non-immune interactions in plate wells, all subsequent reagents include a detergent buffer (0.1% Tween 20). Washing solutions are also prepared with a Tween 20 buffer.

Substrate
The selection of a substrate to make the antibody reaction visible must consider the substrate’s characteristics. The substrate’s exposure to the enzyme must create a soluble, measurable product with low intrinsic absorption that is stable, nontoxic and inexpensive. If a colour change is to be measured using spectrophotometry, a substrate must be chosen that allows the resulting colour to be measured with the available filters. Substrates used with horseradish peroxidase include hydrogen peroxide, orthophenylene diamine, 5 aminosalicylic acid and 3,3,5,5 tetramethylbenzidine (TMB). For alkaline phosphatase, the most suitable is p-nitrophenyl phosphate. The use of PBS as a wash solution can be inhibitory. A TRIS buffer increases the activity of the alkaline phosphatase.

Detection
Every solid phase method has its own method for measuring and interpreting reactions. In spectrophotometric measurement, the optical density of a sample is compared with a calibrator or a limit value. The device may need to be reset against air or the substrate for certain tests of a substrate and conjugate. The calibration curve is calculated based on
dilutions of the standard concentration and their corresponding absorbances. It is usual to use absorbance and the corresponding concentration logarithm. Computer programs are also available, especially for experimental homemade methods. One essential task when introducing new methods and tests is to determine the average absorbance value of samples in the normal population and to set a positive result as 2 to 3 times the standard deviation above this value. Although this method requires the testing of just one dilution of a patient sample, it is not without limitations. This statistical approach cannot be used for parameters that do not have a normal distribution and there may be too few samples for nonparametric statistics. The most significant problem is probably the low reproducibility of absolute values. This problem is tackled by using an “adjusted absorbance value”, where the patient’s absorbance is expressed as a ratio or percentage of a positive control. Even in this case, absolute absorbance values should be monitored to track relative stability between tests. Another approach is to use pooled serum with a given value of arbitrary units and to create a dilution-based calibration curve from which the patient’s value can be read. It is also recommended that the ratio of the areas under the curve be calculated for the test serum and for the reference serum. This technique improves reproducibility and quantitative evaluation but requires more complicated calculations and multiple dilutions of patient serum to obtain values in the linear portion of the curve.

Control and standardisation

The controls for every test include the buffer, a negative control, low and high positive sera and the absorbance of all reagents. In a homemade test, it is important to determine whether the patient’s serum binds to the uncovered plate because in some tests the serum binds non-specifically even if the solid phase is coated with a non-specific protein. This can be a problem in targeting IgM and it is sometimes necessary to test the samples twice – using a bound and unbound tray, and then subtract the results. Whenever a new diagnostic technique is used, it is useful to make control measurements because there can be significant differences between wells and conjugates and it is never possible to guarantee the same laboratory conditions. A standard curve provides more reproducible results. Heterophile antibodies may interfere with sandwich assays. These antibodies may be directed against multiple animal species and could be a cause of a false positive result in the event of the cross-reactivity of two antibodies in the sandwich. Interference can be eliminated by using monoclonal antibodies or non-immune immunoglobulins from a corresponding animal species.
An antibody bound to the solid phase of a well in an ELISA set.

The antigen in the sample binds with the specific antibody in the well.

The secondary enzyme-labelled antibody binds to the bound antigen that is present.

The added chromogenic substrate is broken down by the enzyme attached to the detection antibody and the presence of an antigen in the sample and its concentration can be identified from the colour change and its intensity.

An antigen bound to the solid phase of an ELISA plate set

Antibody in the patient’s serum binds specifically to the antigen immobilised in the well.

An enzyme-labelled antibody against human immunoglobulin identifies the bound human antibody and usually also its isotope.

The added chromogenic substrate is broken down by the enzyme attached to the detection antibody and the presence of the antibody in the sample and its concentration can be identified from the colour change and its intensity.
Capture methods are another way to use solid phase technology to detect antigens or antibodies. Polyclonal or monoclonal antibodies are adsorbed to the solid phase to capture the antigen targeted for detection. The enzyme-labelled antibodies for detection can be monoclonal or polyclonal. Polyclonal antibodies are better suited for detecting varying antigens. Monoclonal antibodies bind only to one epitope. Capture methods are somewhat more sensitive when polyclonal antibodies are used because they capture more antigen epitopes. Capture methods for detecting IgM antibodies against various virus antigens use animal antibodies against the Fc fragment of human IgM adsorbed to the solid phase. There is a risk that all IgM molecules in the patient’s serum will bind to the anti-IgM antibodies regardless of their specificity. If amplification is required, the antigen that should bind to IgM can be conjoined with an enzyme or biotin and incubated with the captured IgM. If IgM specific to the enzyme-conjugated antigen is present, the antigen binds with the patient’s IgM and the enzyme-substrate reaction takes place. The intensity of colour produced by the reaction is directly proportionate to the level of specific IgM antibodies in the patient’s serum. Capture tests have strong potential for IgM antibody detection. The method is effective for detecting antibodies (e.g. for detecting congenital intrauterine infections). Capture methods cannot be used to detect specific IgG antibodies. In the acute phase of infection, especially in the case of congenital virus infections, the majority of IgM antibodies are virus specific.

6.4.7 IgM and IgG separation methods

False positive and false negative results are a relatively common problem in immunoserological tests for IgM. Possible causes of a false positive for IgM antibodies include the presence of rheumatoid factor (RF) in the test serum or polyclonal activation of plasma cells. Rheumatoid factor binds to IgG after IgG binds to the antigen. The process of binding to the antigen causes conformational changes in IgG that expose new antigens on the Fc area of the IgG molecules. IgM RF binds to these newly exposed IgG determinants. If there is no separation, it is impossible to distinguish IgM RF from specific IgM. This distinction is particularly important when testing the serum of new-born children for specific IgM antibodies against the causal agents of intrauterine infections. IgM false positives can also be caused by non-specific antibodies to herpes viruses (EBV, CMV, VZV). Tests can also produce a false negative for IgM antibodies if IgG antibodies inhibit IgM or compete with it for binding sites on antigens. Such false positives and negatives can be avoided by
separating IgG and IgM. It should be remembered that the production of IgM antibodies is usually preserved in patients with compromised cellular immunity (cytostatic therapy, immunosuppressive therapy, biological therapy, in HIV patients and patients with AIDS) because IgM is produced independently of T cells and does not require the presence of cooperating T lymphocytes. It can be an indicator of acute infection or reinfection.

There are several methods for separating IgG and IgM molecules to exclude false positive and negative results in specific IgM and IgG tests, which are based on the molecules’ different size. Physical separation can be achieved using column chromatography or ultracentrifugation in sucrose gradient. Such systems are not practical for clinical laboratories though. Ion exchange columns are effective and widely used. IgG passes through the column and IgM is caught and flushed using a buffer with a lower pH value and higher ionic strength than the application buffer. The quantity of applied serum and wash buffer is controlled to ensure the final dilution is equivalent to the serum dilution. This means that the results are semi-quantitative. In some methods, the serum is adsorbed with staphylococcus protein A to insoluble material. Staphylococcus protein A captures and immobilises IgG molecules at the Fc fragment. Adsorption with protein A eliminates IgG 1, 2 and 4 from the serum, and probably also virus specific IgM. IgG interference is prevented in commercial EIA sets testing for IgM by adsorption with anti-human IgG or aggregated human gamma globulin. Anti-IgG binds with IgG in the sample and prevents IgG from reacting with the antigen. If no IgG binds to the antigen, RF will not bind to the IgG. Aggregated human gamma globulin (AHGG) neutralises RF but does not eliminate the possibility of a false negative result from competition between IgG and IgM for binding sites on the antigen. Anti-IgG processing is more effective that AHGG in eliminating non-specific IgM without affecting specificity. Both methods are still more effective than ion exchange methods for separating IgM from IgG. The methods may not be sufficient to remove IgG antibodies from patients with hypergammaglobulinemia.

6.5 Quality requirements for investigative methods

The value of tests

When a test or method is to be introduced in a laboratory, its implementation should be based on specific criteria. the basic criterion for the use of any procedure is the whether its results can be used in clinically relevant and optimal ways. Other important technical characteristics of every test include specificity, sensitivity, predictive value while the technical
characteristics of results should be characterised by reproducibility, correctness and accuracy.

The clinical or diagnostic sensitivity of a test is measured by the percentage of people having a certain illness for whom the test gives a positive result. A negative result in persons who have the illness is called a false negative and reduces the sensitivity of the test. The specificity of a test is measured by the percentage of persons who do not have the target disease for whom the test gives a negative result. A positive result in such persons is called a false positive and reduces the specificity of the test. Sensitivity refers to the smallest quantity of a detected substance identified by a test. It is also called “analytical sensitivity” Antibody specificity in a test means the ability to distinguish the target antigen from others. (Likewise, antigen specificity means that it captures only a target specific antibody whose production was stimulated by an identical antigen). The predictive value of a test is the probability that the test will correctly detect the presence or absence of a certain disease. The predictive value of a positive test result, or positive predictive value, is the ratio of persons with a positive test result who are true positives, meaning they have the given disease. The predictive value of a negative test result, or negative predictive value, is the ratio of persons with a negative test result who are true negatives, meaning they do not have the target disease. Predictive values are affected by the prevalence of infection in the population.

Sensitivity formula
\[
\text{% sensitivity} = \frac{\text{true positive}}{\text{true positive + false negative}} \times 100
\]

Specificity formula
\[
\text{% specificity} = \frac{\text{true negative}}{\text{false positive + true negative}} \times 100
\]

predictive value formula
\[
\text{% positive forecast} = \frac{\text{true positive}}{\text{true positive + false positive}} \times 100
\]
\[
\text{% negative forecast} = \frac{\text{true negative}}{\text{true negative + false negative}} \times 100
\]
Clinical evaluation of a new diagnostic test is carried out by determining the actual condition of a patient based on clinical criteria or the results of diagnostic tests that can be used for reference. A standard against which a new test is evaluated is known as a gold standard or reference test.

**False positive results**
Enzyme immunoassays for antibodies can give false positive results for various reasons. Rheumatoid factor is produced by IgM anti-immunoglobulin antibodies and causes false positive results for serums containing IgG antibodies in immunoassays where an antigen is bound to the solid phase. For example, serum containing RF and IgG antibodies against the rubella virus can give false positive results in tests to capture IgM antibodies against the rubella virus. The reason is that RF (IgM) binds to IgG antibodies against rubella bound to the antigen adsorbed on the solid phase and they bind with the IgM conjugate. This interference can be prevented by removing IgG antibodies from the sample or by performing an IgM capture assay. Cross-reactive antibodies are the most important cause of false positive results in immunoassays that use an antigen on the solid phase. Cross-reactive antibodies can be removed by adsorption with specific antigens. Another method for removing cross-reactive antibodies is purification in a column containing an antigen. If a test gives false positive results, especially in an area with low prevalence, confirmation tests or supplementary tests must be conducted. This is a frequent problem with recombinant antigens, because antigenic fragments of *E. coli*, antibodies against which are common in patients’ serum, can persist despite purification. Similarly, when testing for Borrelia antibodies, antibodies against flagellin – the flagellar antigen – cannot be taken as proof because antibodies against the flagellar antigens of other gram-negative rods may react non-specifically with flagellin. (A warning sign for this is an isolated find of only flagellin antibodies when testing patients’ serum using the western blot method.

**False negative results**
Imunoassays for antibodies can also give false negative results. Competition between IgG and IgM antibodies can cause a false negative result for IgM if there is limited antigen content in the solid phase and IgG is more successful than IgM at occupying binding sites. Serum samples containing a large number of specific antibodies can cause a prozone phenomenon, where the results are negative at low dilutions and positive at higher dilutions. this situation can be a problem for screening tests, which may require more than one dilution as a result.
Clinical false negative results can also occur if the sample is taken too early in relation to the onset of infection. Knowledge of the natural course of infection and the immune response is important for determining the appropriate time to take a sample for a specific purpose while avoiding a false negative result.

Incorrect laboratory results can be caused by various errors and mistakes, both objective and subjective, at each stage of processing of a sample including the taking and labelling of the sample, its transportation and its handling in the laboratory. There is a risk of confusion at any point in the process of working with a sample; every instrument or aid to use can degrade the sample qualitatively or quantitatively; every worker can influence the quality of the result or its recording or reporting. Therefore, every laboratory result must be evaluated in the context of clinical findings and case history. Any laboratory finding that does not match with a clinical finding or the results foreseen by the physician (the reason why the physician requested testing of the parameter) must be evaluated with caution and in collaboration with laboratory personnel.

Quality control

Quality control is important for ensuring the precision and accuracy of results, and above all the inter-laboratory and intra-laboratory comparability of results. Critical points for quality control are standardisation, continuous monitoring and internal and external control of tests and samples. It is of fundamental importance and should be the focal point of accreditation processes. To determine the optimal concentration of antigen and antibody, block titration is carried out with a reference sample. This titration makes it possible to determine what concentration of reagents provides maximum specific reactivity and minimum non-specific reactivity. The next step is evaluation of the test protocol based on tests of known positives and predicted normal samples from the target population. These studies make it possible to determine the difference between a reactive positive sample and a non-reactive negative sample. This cut-off value is an absorbance value that is set based on the test manufacturer’s specifications, based on a calculation of a certain multiple of the standard deviation of the mean absorbance in the negative population, based on a fraction or percentage of the positive reference value or a combination of all three. No matter how the cut-off is calculated, samples whose absorbance is higher than this limit are positive or reactive while samples whose absorbance is below it are negative. Some manufacturers also indicate a “grey zone”. This approach complicates a test’s interpretation and patient management and for this reason is not recommended. The manual supplied by an instrument’s manufacturer must always be studied
carefully; the instrument must be calibrated and carefully monitored every day during daily maintenance; technical inspections should be carried out at regular intervals. A log must be kept of all monitoring and checking. Every test (or test cycle) must include controls from the manufacturer, own controls and reference samples. Variations in quality or reagent activity between sets or batches are monitored using an external control set, which should include highly reactive, moderately reactive and non-reactive samples and a pool of samples. This external control is used to detect immediate problems, to monitor variation between tests and to track the long-term trend. The values obtained in each test should be monitored graphically to show trends and quality and to provide information for troubleshooting. 

Comparability of results is a very important factor in laboratory quality. As well as inter-laboratory and intra-laboratory comparability, it is important to be able to make ongoing comparisons for an individual patient. At present one can run into problems due to ill-considered methodological changes or the replacement of analysers and diagnostic sets for reasons related to the economics of medical laboratories or corporate lobbying. The inflation in the number of laboratories through absurd and counterproductive competition has led to biological samples being transported hundreds of kilometres to laboratories in other regions, which degrades samples while increasing testing costs. It is practically impossible to compare some markers and serological parameters for a patient whose tests before and during hospitalisation have been carried out by different laboratories, not to mention the effect of changing laboratory diagnostic procedures on the development of their serological profile.

6.6 The significance and use of specific antibody detection

6.6.1 Antibody detection as an indicator of immunity

Professional screening

It is a requirement in some organisations, and sometimes a statutory duty, that before a worker is employed their immunity to certain diseases must be verified. Healthcare facilities in certain countries require immunity against the rubella and varicella-zoster viruses. These viral infections can spread uncontrollably in the non-immune population in the time between exposure to the virus and the appearance of a rash and could be fatal for immunocompromised patients and non-immune adults. Evidence of specific immunity to the hepatitis B virus is required for both healthcare practitioners and students. Although this provides protection against the most common occupational infections in healthcare, there is no reason to neglect anti-epidemic measures preventing the spreading of other blood-transmissible diseases (HCV, HIV, CMV, EBV etc.). In present circumstances, healthcare personnel should be immunised
against all transmissible diseases with which they could become infected, that they have not overcome and against which they can be vaccinated. (Forestry workers, hunters – tick-borne encephalitis, rabies; Vets – rabies; Soldiers, athletes – tetanus and other diseases applicable to the country of activity; Workers abroad – consider the epidemiological situation in the country of activity, the requirements of the receiving country and the profession)

**Preoperative screening**

Current practice in preoperative screening is to check for the presence of antibodies against *Tr. pallidum* and HIV, and the presence of HBsAg. Detection of *Tr. pallidum* and HIV antibodies is important for identifying carriers. For the protection of personnel, it is useful to detect the HIV antigen and HIV antibodies at the same time. Detection of HBsAg is useful for identifying carriers even though it is not sufficient to determine whether a patient is infectious.

**Prenatal screening**

In laboratory practice, the interpretation of the results of serological tests for intrauterine infections can be unclear. The law on health protection requires every pregnant woman to undergo screening for intrauterine infections that could harm the foetus. It is important that test results be interpreted correctly. Prenatal screening can also be carried out selectively when indicated by medical history or risk factors during pregnancy. The risk of intrauterine harm to new-born children is checked by tests for antibodies against toxoplasmosis, rubella, CMV and *Treponema pallidum* bacteria. Alongside detection of HIV or HIV antibodies, a finding of toxoplasmosis or syphilis indicates the possibility of effective antimicrobial therapy to reduce the risk of harm to the child. If HBsAg is detected in the mother, prophylactic immunisation can be provided for the child. The risk of toxoplasmosis is higher when there is contact with a cat; the risk of rubella is increased by contact with non-immunised school age children; CMV infection is a greater risk when in contact with immunocompromised patients and the HIV risk is increased by contact with at-risk groups. The diagnostic methods used are IFA or ELISA. In some countries, rapid screen tests are available in doctors’ surgeries though these may produce false negative results. Nonspecific binding by the fluorescent conjugates in IFA tests can cause false positive results. False positive reactions caused by the presence of antinuclear antibodies or autoantibodies must be differentiated from true positive specific tests. Strong positive antinuclear antibodies can mask the ability to detect specific antibodies
against certain organisms. If the specificity of antinuclear antibodies in a pregnant patient is known, insoluble antigens can be used to absorb the reactive antinuclear antibodies. When using EIA and ELISA methods, the quality of the antigen adsorbed to the solid phase is of vital importance for the quality of the test. Screening tests are usually more sensitive and less specific. Every reactive result must be confirmed by a test of another sample using a higher category of test (a confirmatory test).

Toxoplasmosis
The interpretation of laboratory tests for toxoplasmosis is sometimes very difficult due to the severity of foetal damage in intrauterine primary infection, its chronic course, the availability of multiple serological methods and the nature of tissue parasite infection. It must be handled by an experienced diagnostician. Testing includes use of complement fixation reactions (CFR), which usually require two or more samples to determine the stage. The diagnosis is based on serum antibody titres and their dynamic (increase and decrease during the acute infection period, a steady state in the post-acute period and a further increase on reactivation of disease). At present prenatal screening uses enzyme-linked immunological assays – ELISAs – to detect IgM antibodies with follow-up testing for IgA and IgG in the event of a positive result. An isolated IgM positive with no dynamic and negative results for IgA and IgG is an unexpected finding and suggests non-specific reactivity in the assay. Positive results for IgM and IgA indicate acute disease with the risk of intrauterine damage to the foetus in the event of a primary infection in the first trimester. Positive results for IgM and IgG antibodies indicate a declining acute infection and a comparison of the week of pregnancy and other specific tests can be used to estimate the risk level for the pregnancy. A positive result for IgG in the absence of IgM is a clear sign of post-infection antibodies. The pregnant woman has overcome the primary infection, but it is very probable that Toxoplasma gondii parasites remain in her tissues and the infection may become chronic and produce clinical symptoms in future. There is no risk to the foetus during pregnancy though, and this should be communicated to the mother when presenting a find of isolated IgG.

Hepatitis B
Besides toxoplasmosis tests, pregnant women should be tested for HBsAg at the start of pregnancy and before giving birth. In the first trimester, a HBsAg positive result in a pregnant mother is either a sign of acute infection (if there are positive results for HbcIgM and HBeAg or anti-HBe) or a sign of chronic persistent infection (positive results for HBsAg and HBeAg)
or chronic carrier status and has no effect until birth on pregnancy or the foetus. HBsAg testing in the pre-natal period is important for the safety of childbirth and post-natal care for the child. The new-born child of a mother who is HBsAg-positive must be immunised against Hepatitis B because of the high risk of perinatal, postnatal and breastfeeding transmission. Infections in new-born children have a high risk of becoming chronic and causing hepatocellular carcinoma of the liver at a later time.

Rubella
Serological testing of pregnant women to eliminate the risk of intrauterine infection of the foetus with rubella virus requires precise algorithm and careful interpretation. In our population, anti-rubella vaccination has been mandatory since 1977. There is a tiny number of women of fertile age who have not been vaccinated against rubella or who did not overcome a rubella infection in childhood. Even so, it is impossible to exclude the possibility that there are pregnant women in the population who do not have protective antibodies (women from countries that do no vaccinate, individuals who were not vaccinated, immunocompromised individuals, non-responders). These women are at risk of primary infection with the rubella virus even if collective immunity and the very low probability of the virus circulating in the population means that such a risk is minimal and essentially theoretical. In such an epidemiological situation, it is reasonable that we first test women for the presence of IgG antibodies (testing for protective immunity) and only if this test is negative we test the same sample for IgM antibodies – screening for the mother’s acute infection with rubella virus. Great care must be taken in the interpretation of a positive result for IgM rubella antibodies in the mother. Practitioners must also consider the clinical picture, the epidemiological history and the dynamics of the antibody concentration.

In interpreting serological tests, antibody concentration must be interpreted based on international units or at least with reference to indexes calculated from the ratio of the test’s cut-off extinction value and the extinction value for the patient’s serum. Every off-the-shelf or laboratory-made test has a limit – cut-off – for the concentration of the antibodies in the test, or the achievement of extinction, which constitutes the boundary between a positive and negative result. The range 10% above or below the cut-off value is usually a grey area indicating an unclear result. The cut-off is generally calculated from the values most frequently obtained when investigating large sets. The consideration and evaluation of results close to the grey zone is therefore a sensitive issue. In acute disease, concentrations are high
and clearly indicate the disease. Values close to the cut-off indicate a need for further consultation or tests tracking the dynamic. Serological tests have certain objective and subjective limitations, which must be borne in mind. A false positive result can be caused by cross-reactive antibodies. In some diseases, especially during pregnancy, there is a risk of polyclonal activation of cells leading to a situation in which a test for antigens produces multiple positive results (e.g. IgM for measles, parotitis, rubella) usually in the grey zone or at low levels.

Interpreting the results of prenatal screening tests is demanding and requires good knowledge of antibody production and their dynamics in patient serum at each stage of infection with reference to the specifics of the period of pregnancy. The tests to be carried out are decided by the gynaecologist’s experience, the patient’s clinical picture and legislative requirements. The individual parameters, which the physician often receives from the laboratory with a time lag, provide objective information based on which a decision on next steps must be made and communicated to the patient. Only with good knowledge of the occurrence of individual markers in certain phases of a disease can a gynaecologist inform a pregnant woman with confidence that all is well or that there is a risk of harm. They must bear in mind the fundamental rule to avoid harm both to the body and to mental health and well-being.

Pre-transplant screening
Transplant patients are at risk of a fatal CMV infection that could be transferred accidentally with a donated organ, bone marrow or a blood transfusion when the virus is present in the donor’s cells. Patients can contract a CMV infection from a primary infection, the reactivation of a latent infection or reinfection by an exogenous infection. The immune status of the donor and the recipient of the transplant must be determined before the transplant to ensure that the operation is carried out with seronegative patients. CMV-negative patients receive tissue transplants from CMV negative donors. Blood products given to CMV-negative patients should be CMV-negative.

Vaccination screening
Methods well established in practice for determining an individual’s specific immunity include measuring the serum level of specific antibodies or by skin tests. More recent methods used in research include methods based on the capture of a sub-population of specific antibody-labelled B lymphocytes, the activation of the B lymphocytes to produce specific
antibodies and measurement of the effect of T lymphocytes on the development of the antibody response (e.g. ELISPOT).

**ELISPOT**

The Enzyme-Linked ImmunoSpot (ELISPOT) assay is an adaptation of an enzyme immunoassay to detect and count B lymphocytes producing antibodies against a specific antigen. Peripheral blood cells are counted and placed in the wells of microtitre plates coated with an antigen. The plate is incubated for four hours at 37°C and then washed. Anti-immunoglobulin antibodies and IgG antibodies against human immunoglobulin labelled with alkaline phosphatase are added followed by a substrate (5-Bromo-4-chloro-3-indolyl phosphate) This method is used to test the immune system’s ability to respond to an antigen.

Tests are commonly available for detecting specific immunity after vaccination for the following diseases:

- Tuberculosis using a skin test – tuberculin reaction. To distinguish post-vaccination and post-infection reactions, induration size is measured.
- Diphtheria, based on detection of antitoxic antibodies using a neutralisation assay or immunoassay. The intradermal Schick test can also be used.
- Tetanus, based on detection of antitoxic antibodies using a neutralisation assay or immunoassay
- Pertussis, based on overall agglutinating antibodies or individual classes based on an immunoassay. It is necessary to distinguish between antibodies against the vaccine antigen and the infectious agent antigen. Some (acellular) vaccines do not contain all the bacterial antigens, as was the case with the whole-cell vaccine. This makes it possible to distinguish between post-infection and post-vaccination immunity.
- Polio virus, based on tests for neutralising antibodies when testing for anti-infective immunity. Disease monitoring was based on tests for complement-fixation antibodies.
- Haemophilic invasive disease (Hib infection), based on immunoassays for antibodies against the PRP antigen.
- Measles, based on an immunoassay testing for IgG post-vaccination antibodies
- Rubella, based on an immunoassay testing for IgG post-vaccination antibodies
- Parotitis, based on an immunoassay testing for IgG post-vaccination antibodies
- Hepatitis B virus, based on quantitative immunoassay for post-vaccination antibodies against HBsAg. It is possible to differentiate between post-vaccination and post-infection antibodies.
Hepatitis A virus, based on an immunoassay testing for IgG post-vaccination antibodies.

Tick-borne encephalitis, based on an immunoassay testing for IgG post-vaccination antibodies.

Influenza A and B, based on an immunoassay testing for IgG post-vaccination antibodies.

Pneumococcal antigens can be detected using an immunoassay for IgG antibody groups available in specialised laboratories.

Care must be taken when evaluating individual immunity and it is important to consider the significance of serum antibody concentration alongside vaccination history, the type of vaccination and the administered antigen. Antibodies and memory cells are formed after every contact with a protein antigen. Memory cells are the basis of the immune response even at unmeasurable serum antibody levels. Antitoxic immunity is different. This explains why adults and older persons need a booster when vaccination induces antitoxic antibodies, as in the case of vaccination against tetanus or diphtheria. In contrast to anti-infection immunity based on memory cells, where exposure to either a natural (wild-type) or artificial (vaccine) antigen induces increased antibody production, antitoxic specific immunity needs to be maintained continuously at an effective level because the antitoxic antibodies present in the system are all that the immune system has at its disposal to stop a toxin causing a disease in the initial stage of infection. Memory cells and other components of the immune system would only be able to create elevated levels of antibodies in response to the toxin after it had bound with target receptors and begun to cause clinical symptoms. In the case of tetanus, the minimal concentration of the toxin in the blood means that the disease does not create a specific protective immunity because the antigenic stimulus is insufficient. Patients therefore always need to be actively immunised against tetanus after infection.

6.6.2 Antibody detection in the diagnosis of disease

Acute and convalescent samples - The study of serology has made a significant contribution to the diagnosis of microbial, especially viral, infections in patients. Textbooks state that serological confirmation of a suspected viral disease is impossible without the testing of paired samples. The first sample is taken as soon as possible after the onset of the disease, the second is taken one to two weeks later. These paired samples are referred to as the acute and convalescent samples. To ensure a test is interpreted accurately, the samples are tested simultaneously using the same method for the same test. A more than fourfold increase in the geometric antibody titre or seroconversion – the change from a negative to a positive result –
is considered indicative of acute disease. The requirement of two samples and the related delay in diagnosis and treatment is a disadvantage.

**IgM and IgG specific tests** - Newer enzyme-linked immunoassays that are now available for routine use allow IgG and IgM specific antibodies to be detected from a single sample. A negative result for both IgG and IgM means either no infection or that the test has been conducted too soon. If the early detection of IgM is required, Ig total antibody tests can provide greater sensitivity, as stated above. Commercially available sets are usually designed to test for individual classes. Their correct interpretation must take account of the characteristics of immunoglobulins. IgG antibodies are specific only in the heavy chain region. Total antibodies are antibodies against IgG heavy and light chains that also react with IgA and IgM light chains. IgM antibodies are specific in the heavy chain region.

If IgM antibodies are detectable while IgG is negative, the patient has been exposed to an infectious agent. In certain infections such as toxoplasmosis, early infection can be confirmed by testing for IgA antibodies in the same sample. If there is suspicion of a non-specific IgM reaction, which is possible in the presence of RF, in certain autoimmune diseases or in pregnancy, this can be confirmed or disproved by an additional test of a sample taken later for IgM and IgG. If this test is also IgM positive and IgG negative, it indicates that non-specific reactivity is very likely. Otherwise, seroconversion would have made IgG detectable. This need not apply if the patient has a deficiency in T-cell immunity. A positive result for both IgM and IgG antibodies indicates an infection in the last 3 to 12 months. If IgM antibodies are not present but IgG antibodies are, this is an indication of specific immunity to the tested microorganism. In terms of time, it indicates an infection between 6 and 12 months previously. It is appropriate to use a single sample test for the diagnosis of acute infection if the test system used is specific and sensitive and if the sample is taken at a time when IgM antibodies are detectable (for some tests detecting IgM or total antibodies, this can be 7 to 10 days after infection). The interpretation of test results must also consider the possibility of previous vaccination. After the first dose of a vaccine, IgM antibodies form, and this can lead to confusion between the onset of infection and the onset of immunity – for example in the case of post-exposure vaccination against hepatitis A.

**Comparison of methods** - The transition from CFR to IFAs for the detection of virus antibodies was possible without staff retraining because the results of both tests are given in titres. On the other hand, when transitioning from classical methods using titres for the
quantification of results (CFR, IFA, HIT) to ELISA methods using the ELISA units EU or IU, laboratories must allow a sufficiently long transition period in which samples are tested using both methods concurrently to acquire a representative comparison of the two methods. Another factor that contributes significantly to the success of transition is communication between the laboratory, clinical staff and outpatient surgeries. The preparation of a conversion table is also an essential part of this process.

**Testing of the mother in the event of congenital infection of a child**

If the mother was not screened during pregnancy, there is no need to test her serum after the birth of an infected child. Only if symptoms were documented less than 4 weeks earlier is there reason to test for IgM antibodies. Otherwise the test will not provide relevant information because IgM antibodies have their peak in the first month and need not last longer than 28 days. Detecting IgG antibodies will not contribute to the diagnosis because IgG becomes detectable 2 weeks after infection and can remain present for life. The severity of congenital infections depends on how long the foetus was exposed to infection. A congenital infection can lead to miscarriage, neonatal death, hydrocephalus, mental retardation, blindness, and heart and bone disorders. Some infections produce a range of symptoms (Gregg syndrome in the event of intrauterine infection with the rubella virus). A high percentage of children congenitally infected with the rubella virus, the protozoa *Toxoplasma gondii* or *Tr. pallidum* have no symptoms at birth but develop them gradually over the first 5 years of life. Likewise, serological positivity or negativity for HIV infection of a child can take up to 2 years to determine. If a mother is confirmed to have TORCH immunity at the time of pregnancy, IgG antibodies pass through the placenta and protect the foetus if it is exposed to a microorganism during its development. If the mother is not immune, the microorganism passes through the placenta and can damage the foetus before the mother can develop protective IgG. The foetus responds by producing IgM antibodies, but this response is often slower than in adults. This delayed onset of antibody production allows uncontrolled reproduction of the microorganism, which can be detected serologically after birth. IgM antibodies in umbilical cord blood are always foetal. Unlike IgG, maternal IgM is not able to pass through the placenta because the pentameric IgM molecule is too big. Methods for the direct diagnosis of congenital infections include the cultivation of foetal or placental tissue, demonstration of characteristic lesions or specific neonatal IgM antibodies and now methods based on the detection of genetic information – PCR. Cultivation can take 2-4 weeks; histological changes may not be detectable; or it may not be possible to take a sample. IgM
detection can be a fast and specific diagnostic method. Elevated levels may appear in measurements of aggregate IgM, but specific antibodies provide more information. A basic examination should include testing for aggregate IgM, CRP and specific IgM against TORCH. It is recommended that screening also include testing for RF. It is equally sensitive and more specific compared to testing for aggregate immunoglobulins in the detection of congenital infection. In the event of congenital infection, specific maternal antibodies pass through the placenta and are present in the new-born child’s serum. Because the maternal antibodies bind with antigens present in the test system, false negative reactions can prevent the binding of specific IgM antibodies. A false positive result can occur if RF IgM is present and binds to IgG complexes. Neonatal specific IgM cannot be distinguished from neonatal RF IgM. RF binding can be avoided by removing maternal IgG before the test. This can be done using the same procedures specified above for separating IgG and IgM antibodies.

It is useful to carry out serological tests for antibodies against *Treponema pallidum* in the serum of a pregnant woman in the first trimester. The first sample should detect acute or chronic infection in the woman. Syphilis is the only infection in a pregnant woman that can endanger the foetus not only if the mother experiences primary infection in the first trimester but also if she acquired the infection in the past. A *T. pallidum* infection can be repeatedly reactivated and can transfer to the foetus. In practical terms, this means that one mother can have several children affected by her infection with *T. pallidum* - congenital syphilis. The antibodies that are present are not protective. Prenatal screening includes a test for non-specific anti-cardiolipin antibodies (BWR, VDRL, RRR) and a test for specific antibodies (most commonly TPHA). Anti-cardiolipin antibodies are used for screening purposes despite being non-specific (false positives can be caused by heart disease, rheumatic disease, borrelial infection and some medicines). They can be used to measure the success of therapy because they decrease as tissue repair takes place during treatment of the infection. The antibodies detected by TPHA are specific and a life-long sign of infection. A positive TPHA result in the first trimester is an indication that a pregnant woman should be treated for a high risk of intrauterine foetal damage.

**Syphilis**

Nontreponemal tests for syphilis are the most commonly used screening test. The RPR (rapid plasma reagin) test or VDRL (venereal disease research laboratory) test can be used to detect Wassermann’s (anti-cardiolipin) antibodies. These antibodies – reagin – are produced in response to lipoid material released in large quantities from damaged cells during the early stages of infection and lipids present on the cell surface of the *Treponema* bacteria. Although
these tests are very sensitive, they are not specific. Reactive samples must be confirmed by specific treponemal tests (e.g. TPHA). Prenatal, and in some countries pre-marital, screening tests for syphilis and HIV are mandatory. Syphilis can be treated at any stage and treatment is more successful the earlier it is begun. The reason for prenatal screening is the risk of congenital transmission of the disease. Biological false positives in nontreponemal tests can also be caused by autoimmune diseases. The main value of nontreponemal tests is that they are an effective screening method, they monitor the effectiveness of treatment (their titres fall during treatment and disappear entirely in the event of a cure, in contrast to treponemal tests, which continue to give positive results throughout life) and they can distinguish a treponemal infection from a borrelial infection in the event of a nonspecific positive TPHA result.

**Transplantation and immunosuppression**
The introduction of immunosuppressive treatment that selectively suppresses cellular immunity has increased the number of organs and the volume of bone marrow available for transplantation. Immunosuppressed patients are at risk from opportunistic infections. Bacterial infections can be treated using antibiotics but distinguishing a viral infection from chronic rejection of the allotransplant can be more difficult. Recipients who are seronegative for CMV and HSV before transplantation are at risk. It is necessary to test for IgM against CMV before transplantation and after transplantation almost daily in order to quickly detect infection before transplantation or before the appearance of symptoms or to document the course of an active infection. Although specific IgM does not usually persist for more than 8 weeks, viral IgM against CMV is detectable for several months and years after a documented infection because of its continuous intracellular persistence. Detection of IgM against CMV can be used for the diagnosis of infection in transplant patients. The best method for diagnosing a clinically significant infection is detecting CMV viremia. The absence of specific IgM can be misleading. The timing of sampling is very important for diagnosis. If a sample is taken very early, IgM may not be present in a detectable concentration. If the sample is taken too late, IgM may no longer be present. In some viral infections, specific IgM persists and remains detectable for 12 – 18 months after the onset of infection, so detection of IgG and IgM should be indicated simultaneously for diagnosis of acute infection. This is why patients must be tested for specific IgG and IgM antibodies before transplantation to establish a baseline against which post-transplantation results can be checked.

**Antibodies against certain bacterial infections**
GAS

*Streptococcus pyogenes* - Despite the availability of effective and affordable treatment, group A streptococci remain significant pathogens with immunological consequences. A positive ASLO test is a useful tool for the diagnosis of an invasive infection caused by this microorganism and for confirming a diagnosis of acute rheumatic fever (after pharyngitis) or poststreptococcal glomerulonephritis (after pharyngitis and pyoderma). SLO is produced by some strains of *Streptococcus pyogenes* bacteria and causes a strong antibody response during pharyngitis (85% of infections) and skin infections (25%). Diagnostic methods used to test for ASLO include neutralisations tests, latex agglutination and turbidimetry. An ASLO titre of 200 is the cut-off for a positive result in the classic neutralisation test. Cut-offs for the other methods should be set by inter- and intra-laboratory comparison when introducing the test. The results for different methods are not comparable and every test has its own qualities. Despite the amount of work required and the need for strict adherence to procedures, the neutralisation test is a specific and sensitive method that measures neutralisation antibodies by copying their action in the human organism and permits comparisons between and within laboratories. The anti-DNase test is the best means for monitoring post-streptococcal sequelae after skin infections (glomerulonephritis). DNase is produced by most strains of GAS but the antibody response appears later than in the case of SLO and lasts longer. Anti-DNase antibodies provide better evidence than ASLO for the detection of poststreptococcal chorea minor owing to latency in the development of clinical symptoms. Hyaluronidase is another enzyme. Anti-hyaluronidase antibodies are detected after pyoderma. If patients have late effects of a streptococcal infection and a negative ASLO test, tests for anti-DNase and anti-hyaluronidase should be carried out. These tests should be interpreted alongside other clinical and laboratory assessments. High titres in these tests support a diagnosis of streptococcal infection. If glomerulonephritis is suspected, the C3 and C4 levels can be diagnostic because streptococcal glomerulonephritis provides a trigger for the activation of an alternative complement pathway with the result that C3 is reduced and C4 remains normal.

GBS

*Streptococcus agalactiae* – gram-positive bacteria present in women’s genital tract mucosa – can be a cause of perinatal infection (sepsis, meningitis). Examination investigates the significance of carriage, clinical symptoms of local infection and the presence specific antibodies in the mother. The absence of specific antibodies in the mother and the new-born
child represent a greater risk than the presence of GBS in genital tract mucosa, regardless of whether the mother exhibits clinical symptoms or not.

*Legionella pneumophila*

Antibodies against the cause of legionnaires’ disease can provide evidence of acute infection with this strain of bacteria, or retrospective evidence of infection. IgM antibodies can be detected in the first week after the onset of clinical symptoms of pneumonia. Total or IgG antibodies are detectable between the third and sixth weeks. In some areas legionella bacteria are endemic (the cause of Pontiac fever) and when only IgG is found in a resident of such an area, it is not adequate evidence of infection. In this case it is necessary to obtain direct evidence of acute infection by detecting the antigen in sputum using fluorescence techniques or an IgM positive result or a comparison of IgG concentrations in samples taken during the acute and convalescent stages showing a significant increase in the antibody titre or concentration.

*Rickettsia species*

Working with Rickettsia bacteria is biologically hazardous and tests for direct evidence can only be performed in research laboratories in areas where they are endemic. Serological tests are therefore a suitable method for detecting infections. The Weil Felix test, which uses Proteus vulgaris bacteria to detect cross-reactive antibodies, is now rarely used. Relevant diseases that may require diagnosis in Slovakia include spotting fevers in people coming from areas where they are endemic, or Brill-Zinsser disease (recrudescence of infections acquired in concentration camps or prisons). They are diagnosed based on specific antibodies against antigens from some of the multiple strains of Rickettsia. Methods used include indirect fluorescence, latex agglutination or ELISA assays.

Serological examination for borreliosis

Lyme borreliosis is a disease first diagnosed in 1981 after a cluster of arthritis cases in children in the town of Lyme, Connecticut, was found to have been caused by bites from ticks carrying *Borrelia burgdorferi* bacteria. The manifestation of the disease mirrors the immune response to the microorganism. In its acute stage, LB is characterised by skin lesions known as erythema chronicum migrans (ECM). This is followed by arthritis of the major joints. Neurological and cardiac symptoms can develop later which may not appear to be related to the infection. Some patients with SM were demonstrated to have a high titre for antibodies
against B. burgdorferi of non-specific origin. The diagnostic criteria for LB include an ECM reaction after a tick bite in around 50% of infections. A serological examination is often needed to detect the disease because standard laboratories are not equipped to cultivate samples for testing. Even so, the serological diagnostic method is complicated. Patients can have cross-reactive antibodies in the absence of characteristic symptoms or documented exposure to borrelia. A negative test usually reflects reality. Positive serological findings for borrelia must be interpreted with caution. A rise in concentration after acutely occurring symptoms is strong evidence pointing to infection. On the other hand, the rapid administration of strong antibiotic therapy after contact with a tick, frequently before the appearance of other symptoms, leads to the rapid elimination of the antigenic stimulus in the form of multiplying borrelia. The quantity of specific IgM antibodies is then insufficient for detection. This type of immunity is also unable to prevent reinfection. Diagnosis and interpretation are particularly difficult in the case of chronic patients with symptoms that may be present with LB. Cross-reactive antibodies against other spirochetes can cause incorrect interpretation. LB can cause a specific test for Treponema pallidum (TPHA) to give a false positive but VDRL and WB tests will be negative.

Widal reaction
The Widal reaction is the original test used to detect antibodies against the cause of typhoid, *Salmonella typhi*, specifically Vi (flagella antigen). The reaction was and still is used to detect carriers because of the low capture rate of typical sampling and irregular salmonella secretion in the chronic stage of the disease or in carriers. The term Widal reaction is also used for the detection of agglutination antibodies against other salmonellas or *Yersinia enterocolitica* when studying the aetiology of possible extraintestinal manifestations (Yersinia arthritis, salmonella arthritis etc.). Detection of specific IgM, IgG and IgA antibodies against *Yersinia enterocolitica* is also available with this indication.

Chlamydia infections
Antibodies against chlamydia can be found in a large number of persons exhibiting no symptoms, which limits the evidence value of serological examination. The detection of IgM antibodies in children has been shown to be more relevant and more specific than the detection of antigen in a sputum sample in the case of Chlamydia pneumonia. *Chl. trachomatis* and *Chl. pneumoniae* antigens can be detected by corresponding samples and thereby distinguished. Antibodies against *Chlamydia trachomatis* correlate with the incidence
of gynaecological inflammation in women of childbearing age. *Chlamydia trachomatis* and *pneumoniae* infections can be differentiated based on the specific IgM and IgG against both pathogens and the location of the infection. (on the one hand pneumonia and on the other genital and extragenital symptoms of *Chl. trachomatis* infection)

**Helicobacter pylori**

Despite the availability of direct methods for detecting *H. pylori* (breath test, evidence of antigen in stool – screening tests and biopsy urease test, biopsy material microscopy and analysis of the metabolic properties of a sample to detect urease), serological examination remains useful for its contribution to correct interpretation. Its main benefits are non-invasive sampling and the elimination of risks such as nosocomial infection by endoscopy, false positives when an endoscope is contaminated, and false negatives caused by the use of aggressive disinfectants and incorrect sampling. The existence of cross-reactive antibodies in an insufficiently specific test requires the careful selection of the diagnostic set and the intra-laboratory control. The presence of IgA antibodies indicates acute disease and exacerbation. Concentration does not reflect clinical activity though. IgG antibodies are persistent, and their dynamic is not linked to clinical manifestations.

**Mycoplasmas and ureaplasmas**

The classical cold agglutinin test was formerly an accessible method for detecting a serological response to *Mycoplasma pneumoniae* even though the test gave a positive result for only 50% of documented cases. IgG and IgM antibodies are now available. In a time of regularly recurring epidemics of atypical pneumonias with a typical clinical picture, a finding of specific IgM antibodies is sufficient to confirm the aetiological agent. Latex agglutination sets are available to conduct screening tests for antibodies. Their disadvantages are price and the need for confirmation using ELISA. Their advantages are that they can be used in a clinical setting to determine the correct treatment, skipping ineffective penicillin and cephalosporin primary antibiotics.

**Antibodies against certain viral infections**

Tests to detect specific IgG and IgM or IgA antibodies are available for many viral antigens. For diagnostic and epidemiological reasons, the most common include tests for antibodies against hepatitis, CMV, EBV, RSV, HIV, Parvovirus, flu and preventable viruses. Tests for some less frequent or clinically less well-associated viruses (enteroviruses, polioviruses,
adenoviruses, ECHO, Coxsackievirus) used classical methods CFT, VNT and HIT. These have been replaced by more advantageous immunofluorescence methods. It is also possible to obtain direct evidence by PCR methods. These are also used to confirm the results of other tests or to identify a viral antigen obtained from the isolation of viruses in tissue cultures, chicken embryos or other live models.

**Epstein Barr virus infection**

EBV infection – the primary cause of infectious mononucleosis. It is also associated with Burkitt’s lymphoma and nasopharyngeal carcinoma and chronic fatigue syndrome. The virus is widespread and infects most people during adolescence. IM has a significant peak among young people in puberty (kissing disease). IM is viral tonsillitis with hepatic impairment and a typical find of specifically altered monocytes in the blood. A CRP test can unambiguously exclude a bacterial origin of infection. Tonsillitis, hepatic enzyme activity, negative CRP and blood count are sufficient for the diagnosis of acute IM. The administration of certain groups of antibiotics (ampicillin) may result in a hypersensitivity reaction on the skin (rash), but this is not an effect of allergy to the medicine. Most of the adult population will test positive for IgG antibodies against EBV and therefore a test for heterophile antibodies is sufficient as a screening test for IM. A combination of the Paul–Bunnell, OCH and I.M. tests will detect most infections regardless of their stage. The classic agglutination assays for heterophile antibodies are more specific than the commercially available latex agglutination tests for screening because of the absence of the absorption step that minimises the reaction of anti-Forssman antibodies, which could be a source of false positive reactions. It is currently possible to detect 6 types of antibodies in the serological response to EBV. EBV VCA IgG and IgM, EBV - EA IgG and IgM and EBV EBNA IgG and IgM. Measurement of their dynamic permits a relatively precise determination of the stage and prognosis of EBV infection. EBNA IgM is more sensitive for acute IM infection than testing for heterophile antibodies. EBNA IgG is not present during the acute stage of infection, becomes detectable several weeks or months after infection and then persists for life. Detection of EBNA IgM is diagnostic of reinfection in healthy subjects. Immunocompromised patients are often unable to produce antibodies against EBNA (Epstein Barr nuclear antigen). Another antigen is the viral-capsid antigen (VCA). Antibodies against VCA IgM are strong evidence of a primary infection, especially in young children and for the detection of an atypical course of the disease in adulthood. Primary infection with CMV can present similar symptoms to primary infection with EBV. If there is a negative response for EBV IgM, a test for CMV IgM must be
performed. If patients with IM test negative for EBV VCA IgM, CMV IgM or they have EBV-induced malignancies or if an EBV infection is reactivated in immunocompromised patients, serological methods can be used to detect antibodies against the diffuse or restricted components of EBV early antigen (EA).

HIV
Serological tests for antibodies against HIV 1-2-0 are used in prenatal and preoperative screening. Diagnostic testing has limitations relating to the dynamic in the production of antibodies and the incidence of antigen in the patient’s serum during the disease. Studying only the antibodies has limited information value because the time for seroconversion after infection can be as long as 3 months, and for the new-born children of HIV positive mothers even 6–18 months. The absence of antibodies in an infected person is directly correlated with the incidence of HIV virus in serum and therefore an infection can test negative during viremia early in the disease or when AIDS symptoms have developed. For this reason, it is beneficial to use combo tests which test for both HIV antibodies and the HIV viral antigen, especially for vulnerable groups. Because of the severity of the diagnosis, a positive finding for anti-HIV must be confirmed by the National Reference Centre (NRC). After a sample has been found to be reactive in multiple tests in a local laboratory, a new sample must be taken. If this if found to be reactive, both are sent to the NRC for a confirmation test and if necessary, a new sample of full blood is tested for viremia.

Antibodies against microorganisms associated with tumorous diseases
A test for antibodies against HTLV has recently been introduced because of the risk of T-cell leukaemia/lymphoma being transmitted through transfusion. The detection of a serological response and its interpretation compared to other potential tumour-causing agents such as EBV was discussed above.

The detection of antibodies against HPV is a current subject of discussion with reference to vaccination against this cause of cervical cancer. Tests for HPV DNA can detect current infections and perhaps some latent infections, but a negative result says nothing about the quality of sampling or the temporary or intermittent presence of the virus in cervical cells. It is unclear whether the presence of the virus is sufficient to trigger carcinogenesis or whether it must be persistent and progress through all stages of infection. In the latter case, the development of a type-specific and sensitive test to detect antibodies would be as important as testing for HPV DNA. After testing a prepared vaccine and determining the existence of
suitable L1 and L2 epitopes, it can be expected that serological antibody tests will become more widely available. The interpretation of findings to differentiate post-vaccination and post-infection antibodies and determine their diagnostic and protective value is the subject matter of ongoing studies. It appears that the development of type-specific antibodies can be detected in most women 12 – 15 months after HPV infection. The relationship between antibody development and the prognosis of the infection is unclear. Markers for the post-vaccination testing of immunity or serological markers identifying women at risk of cervical invasion still need to be identified.

6.6.3 Free antigen detection in relation to disease activity
In the course of a disease, free antigens can be found in serum and in other fluids or materials depending on the size and other characteristics of the molecule. Their detection is an important diagnostic step in establishing direct evidence of the cause of infection. For finding evidence of an antigen, the same principles apply as for evidence of antibodies – essentially, the serological reaction of an (unknown) antigen with a known antibody. The presence of soluble antigens in serum is correlated with disease activity.

Bacterial antigens (capsular polysaccharides, cell wall components) can be detected by various methods – double diffusion, counter-electrophoresis, agglutination, ELISA, precipitation. Bodily fluids that are regularly used in tests include blood (serum), CSF and urine. Urine is commonly used for testing because it can be collected non-invasively and it is concentrated in the kidneys, which increases the amount of antigen. A benefit of antigen detection is that it can visualise the presence of killed and non-viable bacteria. Prior treatment with antibiotics inhibiting the growth of microorganisms in cultivation does not affect the result of a rapid screening test for an antigen and it can speed up the administration of suitable treatment. On the other hand, antigen tests cannot be used to evaluate therapy because antigens can persist for a long time even when an appropriate treatment is administered.

Neonatal infections
If no congenital infection occurs, the in-utero environment is sterile. New-born children, especially those born prematurely, are prone to develop infections unless they have sufficient immunity. They are protected by the mother’s immunity, which is of critical importance for the child’s immunity. New-born children are especially vulnerable to infection with Str. agalactiae (GBS) during the first 2 months of life. Detection of the antigen in urine, CSF or
other fluids can be a fast way to establish a diagnosis. Listeria monocytogenes and E. coli infections should be considered in differential diagnosis. A new-born child has practically no defences against infection besides antibodies transferred from the mother through the placenta. The presence of antibodies in the mother therefore provides more valuable information than carriage or the transient presence of microorganisms in the mucosa. Viral infection can be caused by exposure during birth in asymptomatic women with HSV or CMV. If HSV infection is known or diagnosed in the mother’s genital tract mucosa before birth, the child is at high risk of herpes infection. Most CMV cervical infections are asymptomatic and therefore rapid latex diagnostic tests are provided for screening. The mother can also transfer the virus through milk or colostrum so a primary infection with herpes virus or HIV, CMV, HSV or VSV viremia can be an indication to stop breastfeeding.

Bacterial meningitis in childhood
Children aged between 3 months and 2 years are particularly prone to invasive infection by microorganisms with polysaccharide antigens, especially N. meningitidis, Str.pneumoniae and Haemophilus influenzae. B. Rapid diagnosis using latex agglutination permits the timely identification of the bacterial agent, specific treatment and confirmation of the causal agent even if the culture result is negative because of poor transport and handling of the sample after it is taken (refrigeration), or prior antibiotic treatment on previous days. The success of bacterial meningitis diagnosis by latex agglutination and light microscopy depends on the degree of infection in both cases. Both give a clear result only when there are clear and well-developed clinical symptoms. Samples taken too early will be negative. Despite significantly improved diagnostics, 30% of meningitis cases determined, based on WHO criteria, to be definitely or probably bacterial were bacteriologically negative.
Diagnostics of *Str. pyogenes* infection in children

The rapid diagnosis of *Str. pyogenes* (GAS), the cause of bacterial tonsillitis, is a quick and convenient method permitting clear causal treatment indicated because of potential post-streptococcal sequelae and the 100% sensitivity of HSA to PNC. The antigen must be released from bacteria and the inflammatory cells of anti-infective immunity at sites of inflammation. The antigen can then be detected by enzyme immunoassay (EIA) or agglutination. The specificity of the test is higher than its sensitivity and a negative result from a test in an outpatient surgery must be confirmed by pre-treatment culture.

Rapid diagnostic tests for viral antigens have a wide range of therapeutic uses. At present it is mainly immunofluorescence or latex agglutination methods, and in a few cases ELISA and EIA, that are used to diagnose antigens in biological samples (rotaviruses and adenoviruses in stool, HSV and VZV from efflorescence in wards with immunocompromised patients, CMV from biopsy or BAL, HBsAg in serum). Identification of the antigen in legionella pneumonia can be of diagnostic benefit in combination with the clinical picture. Antigen detection does not help to monitor the disease or determine the patient’s prognosis because the antigen can persist for several months after the infection process has been eliminated.
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 function is performed in clinical microbiology departments integrated into the healthcare system. In Slovakia, scientific research is conducted both in specialised research institutions and in universities. 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 that leads 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 diseases 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.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell cytotoxicity</td>
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<td>AHGG</td>
<td>aggregated human gamma globulin</td>
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<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<td>AKAS</td>
<td>Academic library and audiovisual centre (<em>akademická knižnica a audiovizuálne stredisko</em>)</td>
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<tr>
<td>ASLO</td>
<td>anti-streptolysin O</td>
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<td>ATB</td>
<td>antibiotics</td>
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<td>AW</td>
<td>airways</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CDC</td>
<td>Center for Disease Control</td>
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<td>CFR</td>
<td>complement fixation reaction</td>
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<td>CFU</td>
<td>colony-forming unit</td>
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<td>CIE</td>
<td>counterimmunoelectrophoresis</td>
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<td>CMV</td>
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<td>CNS</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>cerebrospinal fluid</td>
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<td>DIC</td>
<td>disseminated intravascular coagulopathy</td>
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<td>dl</td>
<td>decilitre</td>
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<td>diabetes mellitus</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DTP</td>
<td>diphtheria, tetanus, pertussis</td>
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<td>EBV</td>
<td>Epstein Barr virus</td>
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<td>EIA</td>
<td>enzyme immunoassay</td>
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<td>ELISPOT</td>
<td>enzyme-linked immunospot</td>
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<td>EPIS</td>
<td>epidemic information system</td>
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<tr>
<td>ESCMID</td>
<td>European Society for Clinical Microbiology and Infectious Diseases</td>
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<td>EU</td>
<td>European Union</td>
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<tr>
<td>GAS</td>
<td>group A Streptococcus</td>
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<td>GBS</td>
<td>group B Streptococcus</td>
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<td>GLP</td>
<td>good laboratory practice</td>
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<td>HAV</td>
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<td>HBc</td>
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<td>HBsAg</td>
<td>hepatitis B virus surface antigen</td>
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<tr>
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<td>hepatitis C virus</td>
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<tr>
<td>HIT</td>
<td>hemagglutination inhibition test</td>
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