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The first methods for detection of bacterial infections were available around 1880. After staining, bacterial pathogens were recognized in the light microscope because of their size and could be cultivated in culture media. Viruses evaded this approach, as they are significantly smaller, and as obligate parasites are not able to multiply in cell culture media. Although some viral infections could be associated with specific cellular changes and certain depositions in the infected tissue around the turn of the century, e.g. Negri inclusion bodies in nerve cells during rabies, a specific diagnosis was only possible through the development of cell culture methods and modern molecular biology. Today, viral infections can be detected directly by determining the agents, individual viral proteins, or their genetic information, or other materials in the blood of infected people or animals by using appropriate methods. Direct detection of viruses is possible, with the exception of latent or persistent infection forms, only during the acute phase of the disease. In some cases, the pathogens are present in the infected organism only before the symptomatic phase, so the direct

detection of the virus is frequently not successful. Therefore, infections or contact with pathogens is usually demonstrated in virus diagnostics indirectly by characterization of the developing specific immune response.

13.1 How Can Viruses Be Detected Directly?

13.1.1 Viral Cultivation and Derived Detection Systems

For the cultivation and propagation of most viruses, continuously growing cell lines are available today. The preferentially sterile patient material to be investigated, such as blood, serum, pharyngeal lavage or urine, is freed from raw impurities and incubated with the cells in a small volume. After attachment of the virus particles to the cells, antibiotic-containing medium is added to the cultures to prevent the growth of bacteria that may be present as contamination in the test material. In the following days, the cells are microscopically checked for morphological changes, such as the appearance of cytopathic effects, plaques in the cell layer due to cell death, inclusion bodies and giant cells (► [Chap. 5](#)), which allow an initial inference on the replicating virus, and also serve as evidence that infectious viruses were present in the starting material.

The classic form of viral cultivation usually involves a relatively long incubation period of 1–4 weeks. A shortened version is the shell vial assay. In this method, the susceptible cells are incubated with the material that contains the suspected virus in the wells of a microtitre, a 24-well plate or on glass slides in centrifuge tubes and are centrifuged at low speed. This probably alters the fluidity of the cytoplasmic membrane of the cells, thus leading to faster penetration of the pathogens. After incubation for 1–2 days, viral proteins can be demonstrated in the cells by means of immunofluorescence or similar procedures.

Primary cell lines are barely used for cultivation of human pathogenic viruses today. They have only limited division capacity; therefore, they must be repeatedly established. An exception is foreskin fibroblasts, which are occasionally used for cultivation of herpes simplex virus. Particularly, the routine use of embryonic stem cells, which possess increased division capacity, is highly regulated. However, for other primary human cells it must also be demonstrated that they are free of viruses after cultivation, in particular they must not contain any chronic hepatitis viruses (B, C, D) or human immunodeficiency virus (HIV). In veterinary medicine, primary cell lines are commonly used in exceptional cases for the cultivation of certain pathogenic viruses in poultry virology. In certain cases, they are even used for vaccine production as well. This is the case, for instance, for porcine parvovirus.

The formerly widespread viral cultivation in embryonated chicken eggs is no longer used routinely. It is only used in certain cases, such as the cultivation of new influenza virus isolates or for vaccine production (► [Sect. 16.3](#)). By ultracentrifugation of culture supernatants or broken cells, the viruses can be concentrated and rendered available for further investigations. This includes, among other things, electron-microscopic determination of virus particles. Further analyses of viral proteins or genomes serve to characterize the type of virus.

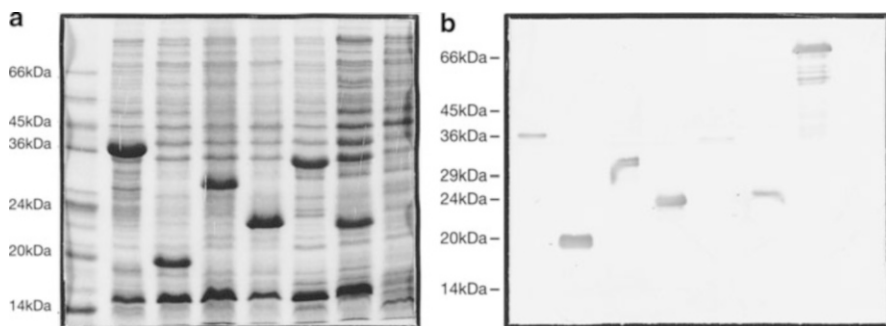


Fig. 13.1 Example for the detection of viral proteins. (a) Sodium dodecyl sulphate–polyacrylamide gel. Protein extracts from *Escherichia coli* bacteria expressing different regions of a protein of parvovirus B19 were electrophoretically separated and stained with Coomassie blue. All proteins present in the preparation exhibit a blue colour (here in *black*). (b) Western blot. The protein bands of the sodium dodecyl sulphate–polyacrylamide gel shown in (a) were transferred to a nitrocellulose membrane (Western blot) and then incubated with rabbit polyclonal antibodies that specifically recognize the parvovirus protein. After a wash procedure, the membrane was treated with secondary antibodies that are conjugated with horseradish peroxidase (immunoglobulins from swine, which are directed against the Fc region of rabbit antibodies) and specifically bind to the bound primary antibodies. Subsequently, the membrane was incubated with diaminobenzidine solutions. In the area of protein bands to which the antibody complexes have bound, a brown precipitate is produced indicating a positive response (Courtesy of Andreas Gigler, Institute of Medical Microbiology, University of Regensburg, Germany)

13.1.1.1 Protein Detection Western Blotting

One way to determine the type of virus is the identification of specific viral antigens in Western blot tests. For this purpose, the proteins of infected cells or virus particles which were previously pelleted by ultracentrifugation are separated by electrophoresis on a sodium dodecyl sulphate–polyacrylamide gel. Their pattern and molecular masses can provide evidence for the type of virus. However, a final assignment is only possible serologically in the Western blot (Fig. 13.1). For this purpose, the separated proteins are transferred from the polyacrylamide gel to a nitrocellulose or nylon membrane (actual Western blot) and then incubated with antisera containing immunoglobulins, which specifically react with the viral antigens of interest (primary antibody). Optimal are preparations of mouse monoclonal antibodies that recognize defined epitopes of a viral protein. Sera from people who have withstood an infection with the corresponding virus type, and thus have produced specifically binding antibodies, can also be used. The nitrocellulose membranes are then treated with other so-called secondary antibodies, which are specific to the Fc regions of the primary antibody; e.g. with immunoglobulins from swine, which were inoculated with Fc regions of murine antibody molecules, and have developed an immune response against murine antibodies. Human antibodies are usually detected with rabbit immunoglobulins, which bind to the Fc regions of human IgG. The different enzymes necessary for the further detection reactions are

covalently bound to the secondary antibody, e.g. horseradish peroxidase. These secondary antibodies interact with the antigen–antibody complexes on the membranes, and in the next step, they are detected by adding the appropriate substrate, in this case diaminobenzidine. In the positive case, brownish protein bands arise (Fig. 13.1). Alternatively, the use of alkaline phosphatase for labelling produces a blue colour.

Antigen ELISA

This variant of ELISA is employed for detecting viral proteins or particles as an alternative to the analytical Western blot. The test is usually performed in microtitre plates which contain 96 wells and are made from specially treated polystyrene. Murine monoclonal antibodies against a specific viral protein are bound to the surface of the plastic wells. Thereafter, suspensions which contain the virus in question or the viral proteins of interest are added to the wells. If the relevant antigens are present, they will interact with the polystyrene-bound immunoglobulins. The antigen–antibody complexes can be detected in the next step by addition of a further antibody, which binds to a different epitope of the same viral protein. These immunoglobulins are covalently conjugated with horseradish peroxidase, so the complex can be visualized by the addition of *o*-phenylenediamine as a soluble substrate. Photometric measurement of the intensity of this chromatic reaction makes possible a quantitative or semiquantitative determination of the viral antigen, which was present in the starting material (Fig. 13.2a). Examples of antigen ELISA applications in human medicine are infections with adenoviruses, influenza viruses, parainfluenza viruses and respiratory syncytial viruses in respiratory secretions and rotaviruses, adenoviruses and noroviruses in faeces. In veterinary medicine, antigen ELISA is used to detect rotaviruses, coronaviruses and parvoviruses in various animal species.

Immunofluorescence

Direct immunofluorescence is used to investigate whether virus proteins are produced in infected cells. The cells are dropped on slides, fixed and treated with alcoholic solvents to render cell membranes permeable. Thereafter, they are incubated with immunoglobulins directed against the viral proteins to be detected. The following treatment with secondary antibodies, which are directed against the Fc region of the previously used immunoglobulins and linked with fluorescent compounds (e.g. fluorescein isothiocyanate), allows one to visualize viral proteins in different compartments, such as the nucleus, the cytoplasm and the cell membranes (Fig. 13.3).

Detection of Virus Properties

Some viruses encode specific enzyme activities, which can be detected as characteristic properties in infected cells, or associated with viral particles in the culture supernatant. These include, for example, the determination of reverse transcriptase activity, which is produced by human immunodeficiency viruses and is a component of the resulting virus particles (► Sect. 18.1). On the basis of the amount of this

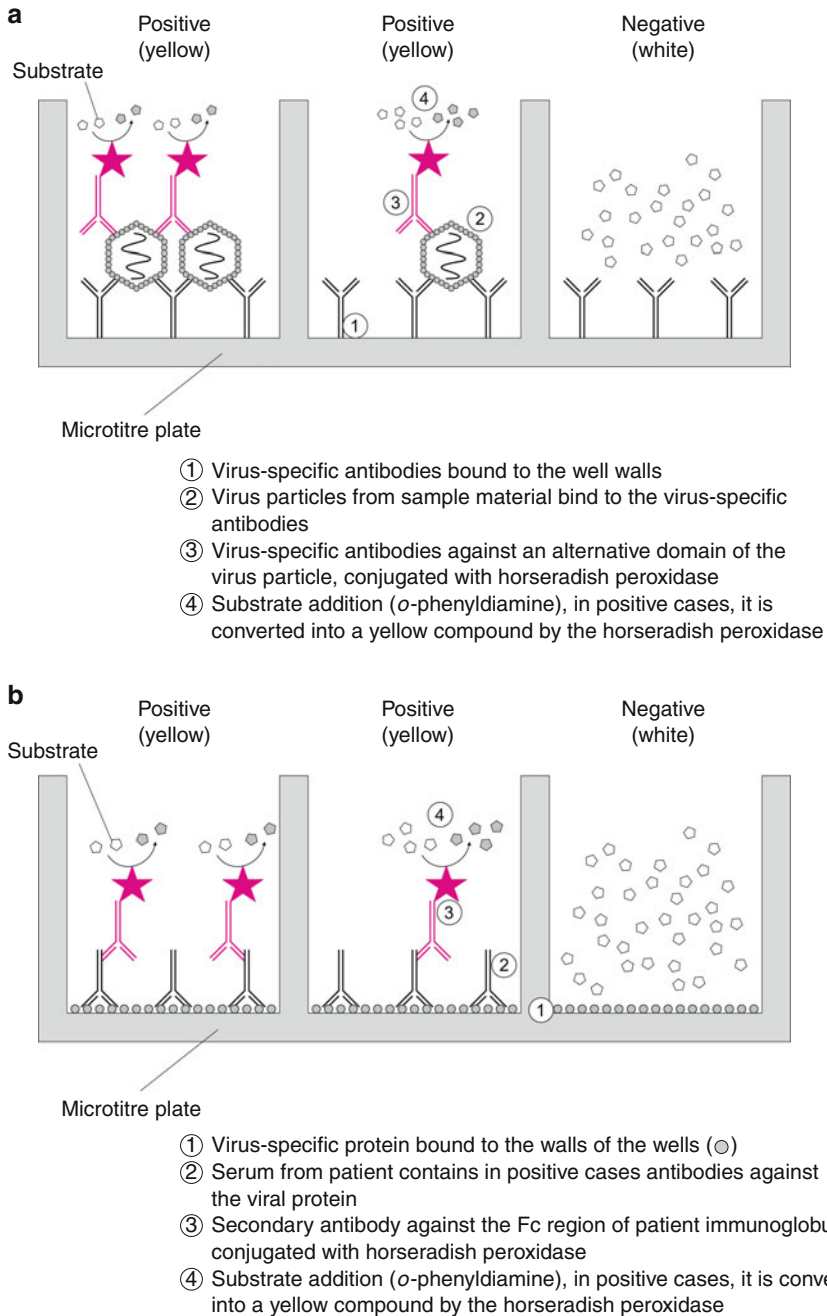
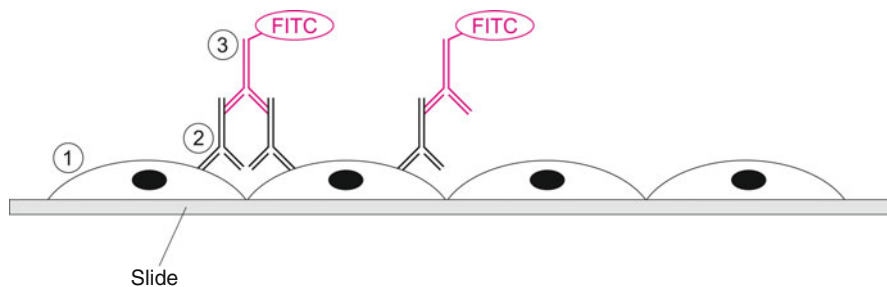


Fig. 13.2 The reaction steps in different types of ELISA. (a) Antigen-capture ELISA for detecting viral proteins or particles. (b) ELISA to detect specific antibodies (Sect. 13.2)



- ① Infected, fixed cells
- ② Specific antibodies, e.g. in sera from patients
- ③ Secondary antibody against the Fc region of human immunoglobulins; if they are bound, the cellular structures that are recognized by the complex are stained in green because of the fluorescein isothiocyanate (FITC) labelling

Fig. 13.3 The reaction steps in the immunofluorescence test

enzyme detected in the culture supernatant, the number of virus particles produced can be quantitatively determined. Other viruses are able to agglutinate erythrocytes. This haemagglutination capacity is found both in human pathogenic and in animal pathogenic viruses. It is associated with viral envelope proteins, and hence with the virions. Therefore, haemagglutination tests can be performed, among others, with paramyxoviruses and orthomyxoviruses (► Sects. 15.3 and ► 16.3) as well as with flaviviruses, togaviruses, coronaviruses and parvoviruses (► Sects. 14.5, ► 14.6, ► 14.8 and ► 20.1). Erythrocytes of appropriate species are used and mixed with the virus-containing suspensions; if red blood cells agglutinate, this indicates the presence of viruses. If the reaction can be inhibited by adding virus-specific antibodies, then this so-called haemagglutination-inhibition test allows determination of the virus type in the starting material. However, this method has become obsolete in routine diagnosis. Nowadays, the haemagglutination-inhibition test is only performed to evidence rubella-virus-specific antibodies in the context of statutory maternity care (► Sect. 14.6).

13.1.1.2 Detection of Viral Nucleic Acids

Alternatively to proteins or enzyme activities, viral nucleic acids can be isolated from infected cells and specifically analysed by Southern blot, Northern blot or dot-blot tests. The purified DNA is cleaved by restriction enzymes. The resulting fragments are separated according to their size by agarose gel electrophoresis. Subsequently, DNA fragments are transferred from the gel to a nitrocellulose or nylon membrane (Southern blot); the same is done for detection of viral RNA (Northern blot), but omitting cleavage with restriction endonucleases. The DNA or RNA preparations can also be spotted directly on the membrane (dot blot). In the case of double-stranded nucleic acid molecules, a denaturation step is required to

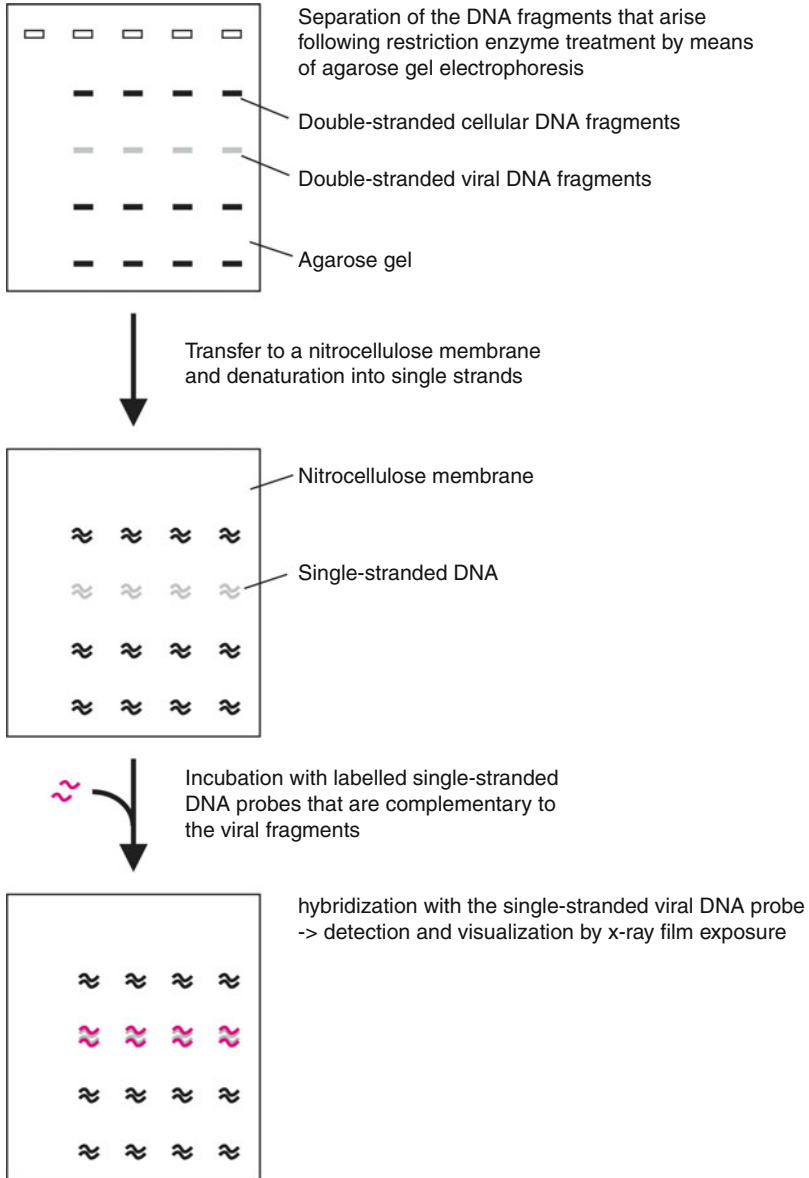


Fig. 13.4 Principle of the Southern blot test

generate single-stranded molecules. Subsequently, the nitrocellulose or nylon membrane is incubated with labelled single-stranded DNA or RNA probes which are complementary to the nucleotide sequences examined and hybridize with them, forming double-stranded molecules (Fig. 13.4). Whereas the hybridization reaction was formerly done using mainly radioactive nucleotides containing ^{32}P or ^{35}S ,

non-radioactive systems are now predominantly used. Usually, modified nucleotides are incorporated into the DNA probes, which were labelled with biotin or digoxigenin molecules. After hybridization with the viral nucleic acids, the nitrocellulose or nylon membrane is incubated with streptavidin- or digoxigenin-specific antibodies. These reagents are covalently linked with enzymes that facilitate detection by a colorimetric reaction. Besides the afore-mentioned horseradish peroxidase (see Sect. 13.1.1.1.1), alkaline phosphatase conjugated reagents are increasingly used. Through incubation with X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) and 4-nitro blue tetrazolium chloride, a deep-purple precipitate is formed as evidence of enzyme activity. The intensity of the colour is directly proportional to the quantity of viral nucleic acid on the blot. Alternatively, the detection reaction can also be performed by chemiluminiscent reagents. As these methods are clearly inferior concerning sensitivity and specificity in comparison with modern polymerase chain reaction tests, these methods are only rarely employed for diagnostic purposes today.

13.1.2 Direct Detection of Viruses in Patient Material

Many of the diagnostic tests used are so sensitive that they can also be used for detection of viruses in sera or other materials from patients without previous *in vitro* cultivation and propagation of the pathogens. This applies especially to infections in which large amounts of virus particles or specific proteins are produced and released into the blood, such as HBsAg in hepatitis B virus infections (► Sect. 19.1), the capsid protein p24 in acute HIV infections (► Sect. 18.1) and the capsids of parvovirus B19 (► Sect. 20.1). In veterinary medicine, the following pathogens are also detected by that means: feline leukaemia virus in the blood of infected cats (► Sect. 18.1.6) and canine parvovirus and bovine rotaviruses and coronaviruses in the faeces of the animals (► Sects. 14.8.6 and ► 17.2.6). The presence of proteins or virus particles can be verified using the methods described in Sect. 13.1.1. Commonly, antigen ELISA is used.

In veterinary medicine, so-called rapid or stick tests have been developed for a number of applications. In such tests, viral proteins are detected by immunochromatographic methods. These tests are based on the principle of an ELISA, but the antibodies are fixed on gold or latex beads (diameter 20 µm) and can be performed in the veterinarian's office. Antibody-loaded beads bind virus particles and/or proteins and migrate, owing to capillary forces, on the membrane, which is submerged in the solution. Antibodies are also fixed on the membrane, and these stop the antigen-loaded beads. These are then visualized as a dark band. Control reactions using immunoglobulin-specific antibodies, which are also bound to the membrane, complement the test (Fig. 13.5). Such rapid tests have been developed, among others, for the detection of various gastroenteritis pathogens in animals (such as parvoviruses, rotaviruses and coronaviruses; ► Sects. 14.8, ► 17.2 and ► 20.1). In human medicine, such simple rapid tests are increasingly used for the diagnosis of influenza virus infections, since treatment with neuraminidase inhibitors follows immediately after a positive result.

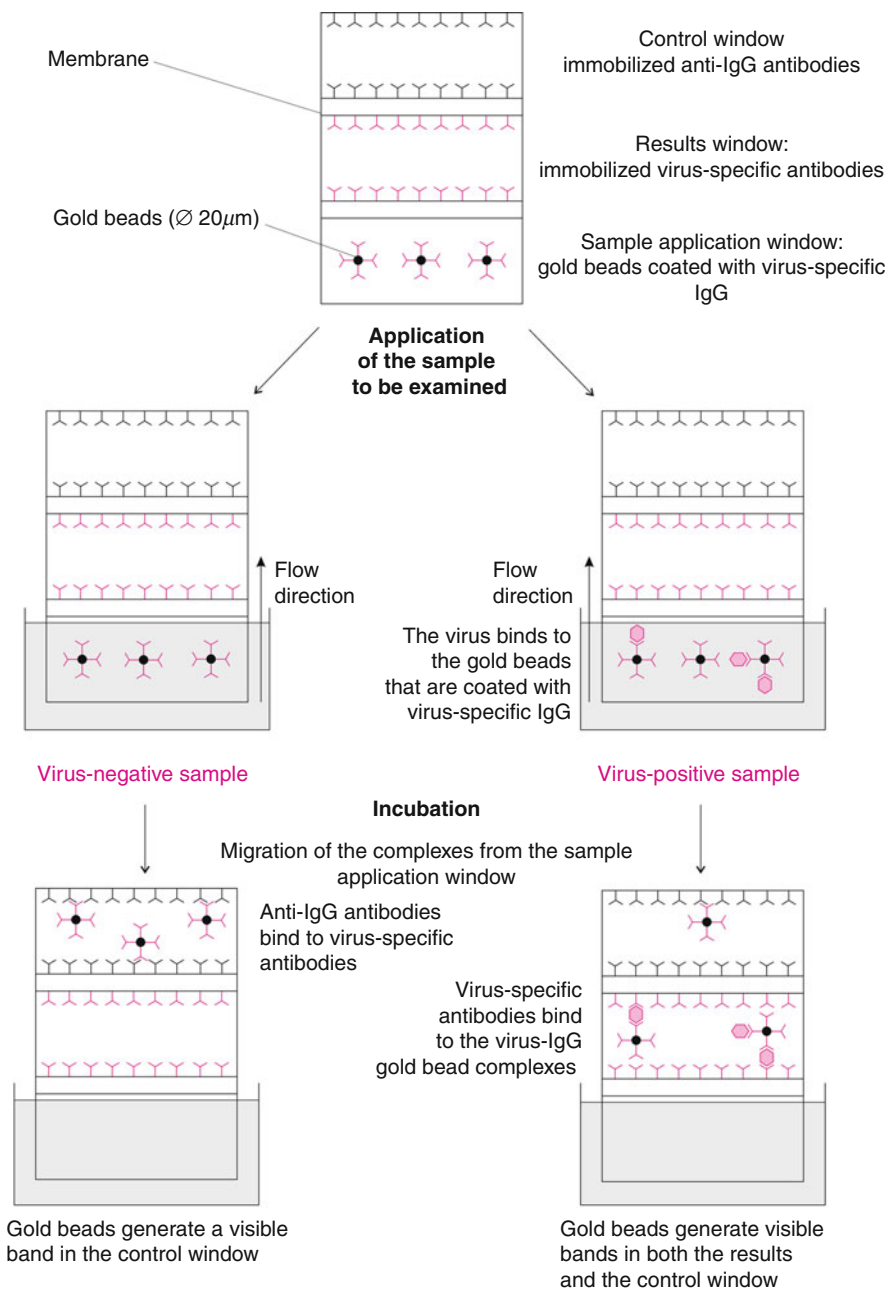


Fig. 13.5 Immunochromatography (rapid test for detection of viruses in patient material). This test is aimed at detecting viruses in liquid patient materials, such as serum, sputum and stool samples. Membranes are used (nitrocellulose or similar) which are coated with specific reagents in different sections (windows). *Sample application window*: large quantities of gold

In recent years, highly sensitive and quantifiable systems have been developed primarily for the detection of viral nucleic acids in the blood or infected fluids and tissues; they have replaced many of the antigen tests, and will be briefly discussed below.

13.1.2.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the amplification of very small quantities of viral genomes or transcripts directly from patient material. Theoretically and practically, it is possible to detect even a single nucleic acid molecule in the test sample. Initially, the viruses have to be broken up (e.g., using chaotropic agents) and the proteins that are present in the sample must be removed, as they can interfere with the subsequent reactions. They are normally degraded with proteases, or removed by phenol extraction and then precipitated and eliminated from the samples by centrifugation. Two oligonucleotides (primer) must be selected (15–20 nucleotides in length) that are complementary to each strand of the double-stranded DNA, flanking a region of 200–400 bases (in real-time PCR also significantly shorter). The DNA is converted into single strands by heat denaturation (usually at 94 °C). Subsequently, the primers are added in high molar excess, and they hybridize with the respective DNA strands during annealing and form short double-stranded regions (annealing, usually at 50–60 °C). The reaction mixture also contains a heat-stable form of DNA polymerase (usually *Taq* polymerase from the thermophilic bacterium *Thermus aquaticus*) and the four nucleoside triphosphates dATP, dGTP, dCTP and dTTP in appropriate concentrations and buffer systems. The hybridized oligonucleotides function as primers. They provide the necessary free 3'-OH ends, onto which the *Taq* polymerase synthesizes the complementary DNA sequence (elongation or chain extension, usually at 72 °C). This step completes the first cycle. As a result, two double-stranded DNA molecules are present in the reaction mixture, which are separated again by a short heat denaturation step, which initiates the second cycle. During the following annealing,



Fig. 13.5 (continued) beads (diameter 20 μm) contain covalently bound IgG antibodies on the surface, which bind specifically to epitopes on the surface proteins of the pertinent virus. *Results window*: IgG molecules are covalently bound to the membrane, and can bind to the same or different epitopes of the virus to be detected. *Control window*: this contains anti-IgG antibodies covalently bound to the membrane. The test principle is as follows. The membrane is incubated with the biopsy material in the area of the application window and placed in a container with buffer solution. If the material contains the virus in question, then the virus will bind to the IgG antibodies on the gold beads. Virus–IgG gold bead complexes and unloaded beads migrate with the buffer front into the results window. IgG antibodies, which are covalently linked to the membrane, bind to free epitopes on the surface of the virus. In this way, the migration of virus–IgG gold beads is stopped, and they form a golden (*dark*) band within the results window. The unloaded (virus-free) IgG gold beads migrate further with the buffer front into the control window, where their migration is stopped, as they react with the immobilized anti-IgG antibodies. As a result, a second band arises in the control window. From the strength of the bands and their ratio, it is possible to infer the amount of virus in the starting material. If the test material does not contain any viruses, only one band will be formed in the control window

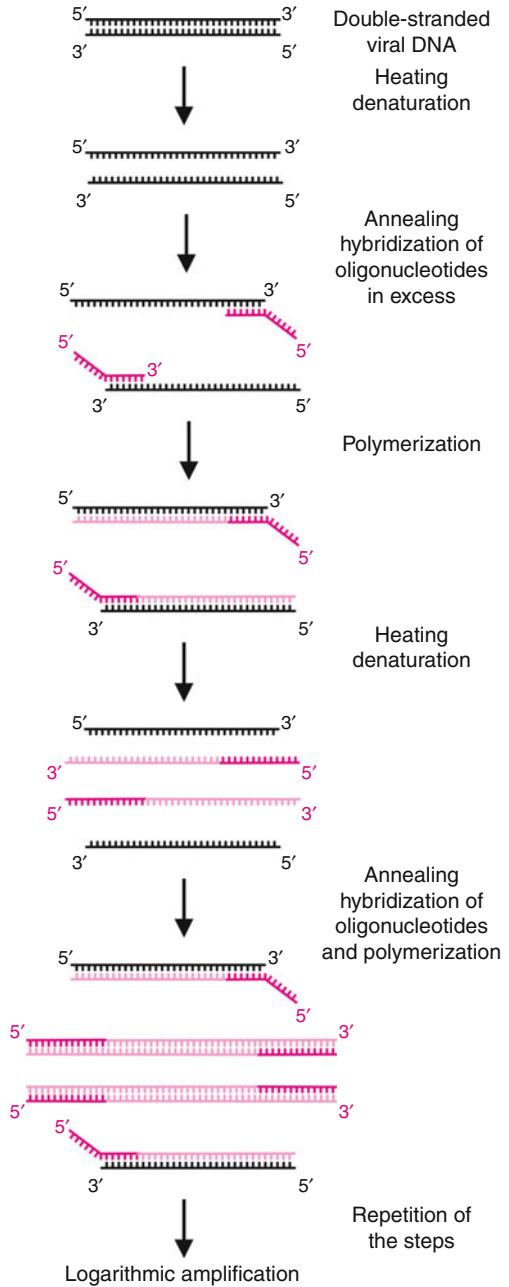
oligonucleotides hybridize again with the single strands and serve as a primer for the synthesis of further double strands: thus, the original DNA molecule has been amplified in a chain reaction to four double-stranded molecules. The cycles are repeated as often as desired, achieving a logarithmic amplification of nucleic acid molecules (2^n , n is the number of cycles; Fig. 13.6). After about 30–40 cycles, the logarithmic amplification phase ends owing to depletion of reagents and enzyme; the PCR amplification products can be separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. For analytical purposes, such as cloning of amplification products, it is also possible to design the primers in such a way that they contain unique restriction enzyme cleavage sites. If the amplified double-stranded DNA is cleaved, the resulting fragment can be identified according to its size after separation of the reaction mixture by agarose gel electrophoresis, or by a subsequent Southern blot assay; or it can be processed further. To increase the diagnostic sensitivity, but also the specificity, two PCRs can be combined by a second, inwardly shifted primer pair (nested PCR). Since DNA is very stable over long periods of time, its sequences can be detected in older and even in fixed tissue samples. Therefore, viruses can be detected in very old, formaldehyde-conserved samples and even in embalmed mummies.

The use of the PCR technique also allows one to investigate how certain virus types, e.g. influenza viruses, have changed and developed in the past centuries. This may provide more precise insights into viral evolution.

If the original nucleic acid is RNA, it is primarily converted into single-stranded DNA using a reverse transcriptase and an appropriate primer. This is followed by the amplification reactions described above. Both reactions can be done in the same reaction tube and using identical buffer conditions. *Taq* polymerase can also be modified so that it can catalyse both reactions. With use of appropriate control standards it is also possible to determine the quantity of nucleic acid in the starting material, e.g. in sera. A disadvantage is often the high sensitivity of the PCR method, particularly nested PCR: since it is possible to amplify even a single DNA molecule, minimal contamination in the reaction mixture is sufficient to generate false-positive results. Besides this carry-over contamination, a primary contamination can occur by infiltration within the laboratory when very high amounts of virus are present in the material (e.g. norovirus or parvovirus B19). This risk is especially high in diagnostic laboratories, where the pertinent materials are routinely handled. Therefore, depending on the PCR application, all solutions, buffers and reaction mixtures which are used for the PCR must be prepared and pipetted in separate rooms (spatial compartmentalization); furthermore, negative controls have to be performed in all tests to indicate possible contamination.

Nowadays, there are automated test systems that allow quantitative determination of viral nucleic acids in the starting material (real-time PCR). For this purpose, the DNA or RNA molecules are amplified as described above. A defined set of single-stranded probes which have a length of 25–40 nucleotides and are complementary to sequences of the amplified regions are added to the reaction mixture. These sequence-specific probes are labelled with a fluorescent group at the 5' terminus (e.g. 6-carboxyfluorescein). Conversely, they carry a different chemical

Fig. 13.6 Principle of the polymerase chain reaction



group at the 3' end (e.g. 6-carboxytetramethylrhodamine) which suppresses the fluorescence of 6-carboxyfluorescein. Prerequisite for this quenching effect is that the two groups are located close to each other, e.g. at the two ends of each probe (fluorescence energy resonance transfer technology). If viral genomes are present in the sample, then the nucleotides of the probe are degraded by the exonuclease activity of *Taq* polymerase during the polymerization process. This step leads to separation of the two fluorescent groups. The quenching effect is abolished by this step and the fluorescence of 6-carboxyfluorescein can be measured in real time. The more viral nucleic acid was in the starting material, the higher is the fluorescence intensity. Common procedures have a logarithmic amplification efficiency of six to eight logarithmic magnitudes. With use of several primer pairs and differentially labelled probes, various amplification products can also be detected specifically and quantitatively in a single assay (multiplex PCR). Thus, internal controls are possible for checking both the DNA extraction efficiency and possible internal inhibitions. Another advantage of real-time PCR is the significantly reduced risk of end-product contamination.

Alternatively, the amplified DNA sequences can be quantitatively detected in an ELISA-like hybrid capture assay. The amplification products are extracted from the solution by a specific probe that is bound to the polystyrene microtitre plates. If biotinylated primers or nucleotide derivatives are added to the amplification step, then they can be detected by peroxidase-conjugated antibodies.

13.1.2.2 In Situ Hybridization

In frozen sections of infected cells or tissues, such as in pathology, viral DNA and RNA can be detected by in situ hybridization with specific, labelled DNA or RNA probes that are complementary to the target sequence. Usually, the probes are labelled with ³H-thymidine or biotinylated nucleotide derivatives. In the first case, after hybridization, the frozen sections are coated with a film emulsion and subsequently developed, whereby a granular blackness can be observed in the infected cells by microscopic examination. This method can be combined with PCR, so even minute amounts of viral nucleic acids can be detected in frozen sections (in situ PCR).

13.1.2.3 Branched DNA Detection

This test system is suitable for the detection of nucleic acids in sera of patients. It was developed to determine the quantity of DNA genomes of hepatitis B virus as well as the amount of RNA genomes of HIV and hepatitis C virus. The proteins that are present in the samples are degraded by protease treatment, and DNA as well as RNA molecules are concentrated by centrifugation. DNA oligonucleotides are added to the solution as primary probes, which are partially complementary to viral genome sequences and form with them short double-stranded regions; the non-complementary part of the oligonucleotide remains as a single strand. This nucleic acid complex is bound to microplates containing oligonucleotides as capture probes

in polystyrene wells and that are complementary to other regions of the viral genomes. In the next step, DNA molecules are added, and they hybridize through their ends with the single-stranded regions of the primary probes and have about 20 branches, whose sequences can hybridize with further complementary probe oligonucleotides. These probes are labelled with alkaline phosphatase. The quantitative detection is performed by addition of substrates which release chemoluminescent molecules during the enzymatic reaction. Inasmuch as the original sequence is not amplified in the branched DNA detection method, but the signal is, it is less vulnerable to contamination.

13.2 How Are Specific Immune Reactions Used for the Indirect Detection of Viral Infections?

As already mentioned, viruses are present and can be detected often only for a short time in patients. Therefore, a diagnosis must frequently be made indirectly, i.e. by determining the immune response that is elicited against a given pathogen during infection. Usually, patient antibodies are detected which specifically bind to specific viral proteins in the serum, and sometimes in the cerebrospinal fluid. IgM antibodies generally indicate an acute or recent infection. By contrast, if IgG antibodies against a specific virus are detected, a past or former infection can be inferred. They are also indicative of an immune status which protects the person from a new infection with the same pathogen (► [Chap. 7](#)). Especially for diagnosis of acute infections, it is important to determine the concentration of IgM and IgG antibodies during infection. Occasionally, one also tests for IgA antibodies. All antibodies can be detected by Western blot, ELISA or indirect immunofluorescence analyses. Sometimes, the haemagglutination-inhibition test is also used. If specific functions are to be associated with immunoglobulins, such as their ability to neutralize the corresponding virus, then one examines whether the immunoglobulins can inhibit the infection *in vitro*. For this purpose, specific quantities of infectious virus particles are incubated with antibodies before the mixture is added to the cells of a tissue culture ([Sect. 13.1](#)). Neutralization tests are still routinely used for detecting antibodies against poliovirus and in veterinary virology and for the detection of antibodies against canine distemper virus.

13.2.1 Western Blotting and ELISA

Western blotting and ELISA differ from the tests described in [Sect. 13.1](#) in one point: in this case, the antigen is predefined, i.e. certain viral proteins or particles. They are separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Western blot). In ELISA, the protein preparations are bound to the polystyrene of microtitre plates or other matrices. The patient sera in which the antibodies in question are to be detected are incubated with the nitrocellulose membrane or pipetted into the wells of microtitre plates.

In this case, the detection reactions are also performed with enzyme-conjugated secondary antibodies that specifically recognize and bind to the Fc region of human IgM or IgG (Fig. 13.2b). ELISA does not only allow an answer to the question of against which viral antigen the antibodies are directed, but also an answer to the question of the concentration of immunoglobulins, as serial dilutions of sera can be analysed. Until a few years ago, viruses cultivated and enriched in cell culture were commonly used as antigen material in Western blotting or ELISA. However, viral proteins are preferentially used today, and they are produced using genetic engineering methods. For this purpose, viral antigens are chosen against which virus-type-specific antibodies are produced during infection and that are also conserved and hence not easily mutable. Usually, they are viral structural proteins. The genes that code for these proteins are cloned and expressed in *Escherichia coli* or yeast under the control of bacterial promoters. These bacteria produce large amounts of the respective viral proteins, which can be purified and used as antigens in Western blotting or ELISA. This approach has proven to be cheaper and less hazardous than the cultivation of large amounts of infectious pathogens. It is also known that antibodies are regularly produced against specific epitopes in the course of infection; these protein regions can be synthesized chemically as peptides and used as antigens in ELISA.

The use of purified protein preparations makes the electrophoretic separation of proteins dispensable. In these cases, they are directly applied on the membranes with a brush or a fountain pen. In these line-blot tests, the same membranes are used as are employed in Western blot tests.

13.2.2 Indirect Immunofluorescence Tests

For this purpose, in vitro infected culture cells are deposited and fixed on slides. The serum dilutions to be tested are added to the cells and bound antibodies are detected by using fluorescein isothiocyanate conjugated immunoglobulins that, depending on the question to be answered, are directed against IgG or IgM of the species examined.

13.2.3 Test Systems for Detection of the Cellular Immune Response

For several years, attempts have been made to obtain additional diagnostic or prognostic information by detecting specific cellular immune responses. The test systems used are considerably more complex than those employed for the detection of specific antibodies. In the first step, T lymphocytes must be isolated from the blood of the subjects by density gradient centrifugation (e.g. Ficoll gradient) or enriched by lysis of erythrocytes. The further purification of the different cell populations is performed by binding of cells to magnetic beads coated with specific

antibodies (e.g. directed against CD4 or CD8 receptors on the surface of T-helper cells and cytotoxic T lymphocytes). Alternatively, the cells can be labelled with specific antibodies directed against certain surface proteins and then isolated by fluorescence-activated cell sorting (FACS). In the second step, the respective T-cell populations are analysed in tests.

In addition to intracellular staining for detection of cytokines in the cells by FACS analyses, the tetramer test is used when specific T cells which recognize viral peptides in complex with MHC class I antigens are to be detected. The procedure relies upon recombinantly produced MHC class I or MHC class II proteins which are linked with biotin molecules in their carboxy-terminal domains. After their purification, the MHC class I protein preparations are incubated with β_2 -microglobulin and peptides (in the case of MHC class II proteins only with peptides) that are derived from the viral antigens against which the T lymphocytes react that one wants to detect. In the next step, the peptide–MHC–biotin complexes bind to avidin, which is labelled with fluorescent dyes (e.g. fluorescein or phycoerythrin) and has four binding sites for biotin. The fluorescent avidin–biotin–peptide–MHC complexes are then incubated with purified T lymphocytes, which were isolated from the blood of the subjects examined (Fig. 13.7). If T cells whose T-cell receptors specifically bind to the MHC–peptide complexes are present in the cell preparation because of a past infection, then the fluorescent complexes will bind to the surface of T cells, which can be quantitatively detected by FACS analyses.

The lymphocyte proliferation or stimulation test is used for detecting specifically reactive lymphocyte populations. In this procedure, the isolated lymphocytes are incubated with the antigens in question or with antigen-presenting cells. Lymphocytes, which recognize the antigen in question, begin to proliferate. Lymphocyte proliferation can be detected by addition of ^3H -thymidine to the culture medium and can be followed by measuring the incorporation of the radioactive nucleotide into the cellular DNA. Alternatively, secreted proteins (such as certain interleukins), which are released as a result of lymphocyte recognition, can be detected by ELISA in the culture supernatant or by means of enzyme-linked immunosorbent spot tests at the level of individual cells.

The enzyme-linked immunosorbent spot test is used for the quantitative determination of lymphocytes, which produce and secrete certain proteins (such as interleukins, chemokines and antibodies) as a result of specific antigen recognition (or repeated stimulation). If the detection of interleukins or chemokines is desired, then antibodies which specifically bind to the respective proteins are attached to the polystyrene material of microtitre plates; if specific antibodies are to be detected, then the microtitre plates are coated with the corresponding proteins. The lymphocytes to be examined, which were isolated from the blood of subjects, are added to the wells of the microtitre plate so that they form a thin layer on the floor. During an incubation period at 37 °C, the lymphocytes secrete the products in question, which bind to the interleukin-specific antibodies or to the protein antigens on the floor of the microtitre plate.

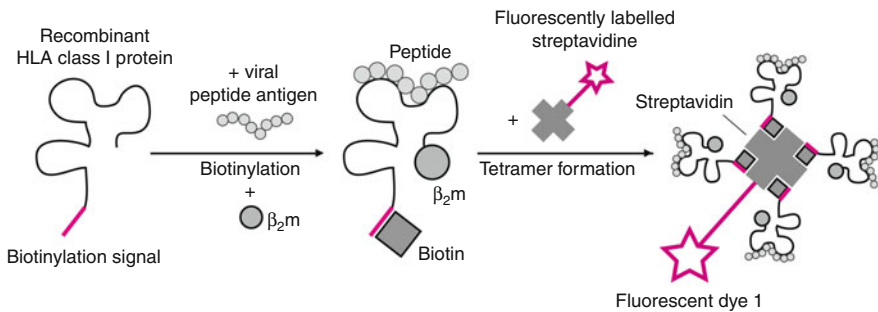
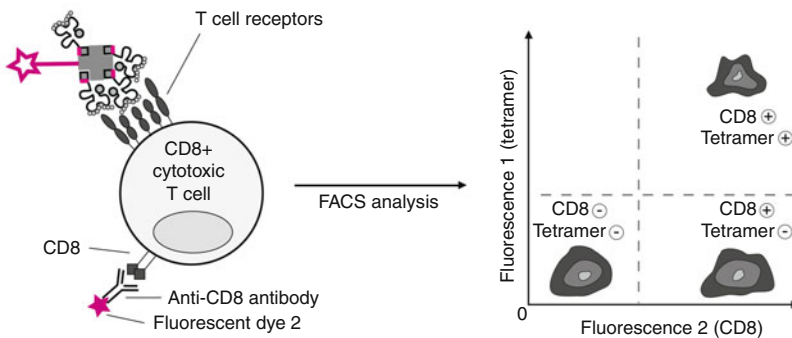
a Production of tetramer molecules**b Detection of epitope-specific T cells by flow cytometry**

Fig. 13.7 Course of the tetramer test for detection of specific reactive T lymphocytes. (a) Production of tetrameric molecules. Recombinantly produced HLA class I antigens are biotinylated at a carboxy-terminally attached biotinylation domain and incubated with a virus-specific peptide antigen (T-cell-specific epitope) and recombinantly produced β_2 -microglobulin (β_2m). Four biotinylated HLA class I/peptide/ β_2 -microglobulin complexes bind to streptavidin molecules, which were labelled with fluorescent dye 1, forming “tetramers”. (b) Detection of virus-specific T cells by flow cytometry. CD8⁺ T lymphocytes are isolated from the blood of patients and incubated with CD8-specific monoclonal antibodies, which are labelled with another fluorescent dye. The labelled CD8⁺ T cells are incubated with the tetrameric complexes. If T lymphocytes are present which recognize the viral peptide antigen, they will bind to the tetrameric complexes using their specific T-cell receptor. On the basis of their fluorescent labelling, the T cells that are linked to the tetrameric complexes can be measured, quantified and distinguished from those without complexes by fluorescence-activated cell sorting (FACS) analyses

Subsequently, the lymphocytes are removed and the plates are washed. A semisolid agar layer is added containing dye-labelled antibodies, which recognize the complexes formed. Each of the relevant antibodies or interleukin-producing lymphocytes is depicted in a perceptible colour point on the floor of the plate. Counting is usually performed by appropriate automated camera and evaluation systems.

13.3 What Important New Methods for Detection of Viruses Have Been Developed in Recent Years?

13.3.1 Multiplex Reactions and Genotyping

In contrast to the general detection of bacterial infections, e.g. by amplification of bacterial 16S ribosomal RNA, no general screening test is available for detecting viral infections. This implies searching specifically for each potential pathogen. With regard to practicality and costs, the ability to detect multiple viruses using a single PCR would be a solution. Ideally, all possible pathogens for diarrhoea or meningitis could be detected in a single assay. The principle of multiplex PCR takes this into account, as several primer pairs and probes can be mixed together. However, this approach is generally associated with a loss of sensitivity. An elegant solution is when a conserved region can be amplified with a primer pair, in which the determination of subtypes or genotypes is possible by different probes with a sufficient number of sequence differences. This principle has been successfully applied in determining the high-grade and low-grade malignant subtypes of human papillomaviruses. On the basis of microarray chip technology, efforts are currently being made to develop miniaturized chip tests for viral diagnostics.

13.3.2 Resistance Tests

In the treatment of persistent viral infections, such as with HIV, hepatitis B virus, hepatitis C viruses, herpes simplex virus and cytomegalovirus, we are confronted with the problem of the development of resistant viruses. It is often important to search specifically for the presence of well-known mutations which determine the respective resistance. In the case of HIV, phenotypic tests were initially applied in which the virus infecting the patient was cultivated in cell culture and examined for sensitivity to the corresponding drugs. Later, it was attempted to insert the tested genes in recombinant viruses. But, it is more practical to determine the predominant genotype of the virus, e.g. by PCR and subsequent sequencing. In the case of HIV, the genes encoding the reverse transcriptase complex and the viral protease are examined. Owing to the increasing prevalence of already resistant viruses in new infections, this can also be important before the first therapy.

13.3.3 Biosensors

In recent years, biosensors have been increasingly discussed and tested as rapid and sensitive test systems for the detection of various classes of substances and molecules. The immunosensors allow the measurement of specific antigen–antibody complexes; they are based on different principles, which can be addressed herein only briefly. For use as chemical sensors in liquids, such as sera, volume resonators seem to be particularly appropriate today. These include oscillating quartz crystals,

which (depending on the test principle) are coated with antigenic proteins or monoclonal antibodies on a specially treated surface. If an alternating electric voltage is applied to these quartz crystals, the crystal becomes excited, resulting in elastic oscillations, whose amplitude reaches a maximum when the electrical frequency matches one of the natural mechanical frequencies of the respective quartz crystal. These oscillations can be measured by appropriate measuring systems. If a quartz crystal coated with antigens is placed in a solution containing specifically binding antibodies, then they bind to its surface and alter its mass. This changes the oscillation frequency, indicating positive antibody detection. Besides these piezoelectric immunosensors, attempts are being made to develop measuring techniques that work similarly to the potentiometric electrodes of pH metres. In this case, the objective is to determine the potential change that arises owing to the antigen–antibody complex formation on a thin, equilibrated silica gel layer on the surface of the pH glass membrane. Ion-sensitive field-effect transistors are a subgroup of potentiometric immunosensors; they have the fastest response time (only a few seconds) among all the systems mentioned, thus making possible extremely fast measurements.

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