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# Diagnostic Techniques: Serological and Molecular Approaches<sup>☆</sup>

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## Glossary

**EIA** Enzyme immunoassays are methods used to estimate virus-specific IgG and IgM antibodies or virus antigens by enzyme-labeled conjugates.

**PCR** By the polymerase chain reaction (PCR) and with specific primers, DNA sequences can be multiplied.

**RT-PCR** For RNA, nucleic acid has to be transcribed with reverse transcriptase (RT) enzyme to complementary DNA prior to PCR.

## Introduction

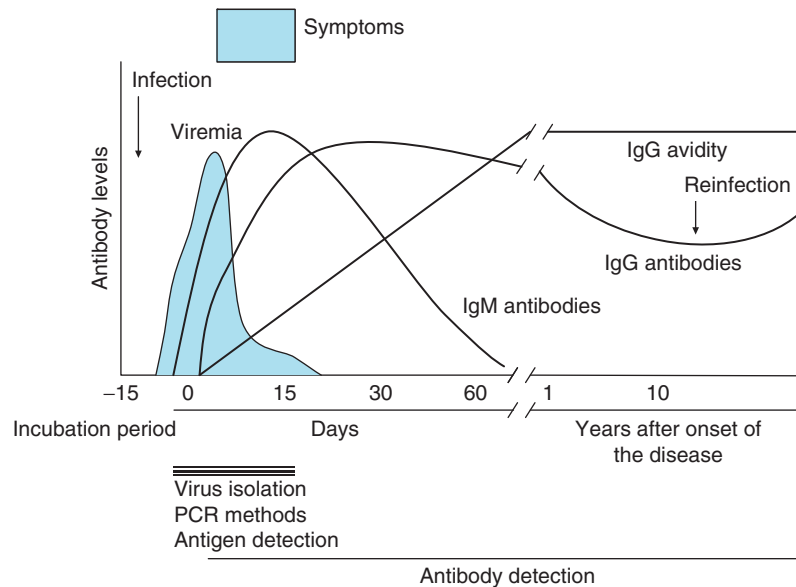
Specific virus diagnostics can be used to determine the etiology of acute viral infection or the reactivation of a latent infection. Two approaches can be used: demonstration of a specific antibody response or of the presence of the virus itself. Nucleic acid testing has become the main approach for the demonstration of the presence of virus while cultivation is used by fewer specialized laboratories and antigen detection methods have moved to the point of care. Serological methods are used for measuring the antibody response caused by an active infection.

In this article, we briefly describe the principles of the most important serological methods and molecular applications that are used to provide information about the viral etiology of the clinical condition presumed to be caused by a viral infection. The diagram of the course of acute virus infection ([Figure 1](#)) indicates the optimal methods for viral diagnosis. Following transmission, the virus starts to multiply and after an incubation period clinical symptoms appear with simultaneous shedding of infectious virus. The presence of infectious virus or viral structural components can be investigated directly from various clinical specimens either by nucleic acid detection assays, virus isolation, or antigen detection assays. Irrespective of the method of direct virus detection, specimens collected at the site of symptoms give the most conclusive diagnosis.

Virus-specific antibodies appear somewhat later (from some days to weeks, called a window period). When the virus-specific antibody production reaches the level of detection, at first immunoglobulin M (IgM) antibodies and some days later immunoglobulin G (IgG) antibodies appear, and the amount of infectious virus starts to decrease. If this is the first encounter with this particular virus, that is, a primary immune response, IgG antibody levels can stay at a relatively low level, whereas in a later contact with the same antigen, that is, in secondary response, IgG levels increase rapidly and reach high levels while IgM response may not be detectable at all. Antibodies are usually investigated from serum samples taken at acute and convalescent phase of the infection. In selected cases other materials such as cerebrospinal fluid and other body fluids can also be analyzed.

In order to reach the best diagnosis for each patient, it is important to select the most suitable method using the right sample collected at the right time.

<sup>☆</sup>*Change History:* December 2014. M Waris updated Abstract, Introduction, and the following sections: Principles of Serological Assays, Immunoassays, Immunoblotting, Detection of Viral Antigens, Nucleic Acid Detection Assays including a new paragraph, Point-of-care Tests including a new paragraph, Future Perspectives, and Further Reading.



**Figure 1** The course of virus infection. The shedding of infectious virus after the incubation period and typical antibody response. Recommended diagnostic laboratory methods have been marked.

## Principles of Serological Assays

During most primary infections IgM antibody levels peak at 7–10 days after the onset of illness and then start to decline, disappearing after some weeks or months. An IgM response is usually not detected in reactivated infections or reinfections. The production of IgG antibodies starts a few days after IgM response and these antibodies often persist throughout life.

Serological diagnosis is usually based on either the demonstration of the presence of specific IgM antibodies or a significant increase in the levels of specific IgG antibodies between two consecutive samples taken 1–4 weeks apart. The antigen for the test can be either viable or inactivated virus or some of its components prepared by virological or molecular methods. Isotype-specific markers or physical separation are used to demonstrate the isotype of the reacting antibody. In some cases, even IgG subclass specificities are determined although they have limited value in diagnostic work.

During the early phase of primary infection the specific avidity of IgG antibodies is usually low but it increases during the maturation of the response. Diagnostic applications of the measurement of the avidity of IgG antibodies against specific antigens have been developed to help distinguish serological responses due to acute infections from those of chronic or past infections.

Serological assays are useful for many purposes. In primary infections they often provide information about the etiology even after the acute stage when infectious virus or its components can no longer be demonstrated in the samples. They are widely used for screening of blood products for the risk of certain chronic infections, evaluation of the immune status, and need for prophylactic treatments in connection with certain organ transplantations. Serology may also be used to confirm an acute infection in cases when the virus can be asymptotically present. They are also widely used for epidemiological studies, determination of vaccine-induced immunity, and other similar public health purposes.

Serological assays have their limitations. In some infections the antibody response is not strong enough or the limited specificity of the antigens used in the assay does not allow unambiguous interpretation of the results. In infections of newborns the presence of maternal antibodies may render the demonstration of the response in the baby impossible. In immunocompromised patients the serological response is often too weak to allow the demonstration of specific responses. In these cases other virological methods should be considered.

Other clinical specimens than sera can be used for antibody assays. IgM and IgG antibody determinations from cerebrospinal fluid are used for diagnosis of virus infections in the central nervous system although new molecular methods are increasingly replacing them. Recently, increasing attention has been given to the use of noninvasive sample materials such as saliva or urine. They are becoming important for public health purposes but their value for diagnosing individual patients is still limited.

## Principles of the Most Common Serological Tests

### Neutralizing Antibody Assay

Antibodies that decrease the infectious capacity of the virus are called neutralizing antibodies. They are produced during acute infection and often persist during the entire lifetime. They are also useful as an indication of immunity. Both IgM and IgG antibodies participate in the neutralization.

In the assay, known amounts of infectious virus are mixed with the serum sample and incubated for a short period after which the residual infectivity is measured using cell cultures or test animals. This infectivity is then compared with the infectivity of the original virus and the neutralizing capacity is calculated from this result. Today, neutralizing antibody assays are often done by plaque reduction assays with better accuracy but with somewhat more complex technical requirements.

Neutralizing antibody assay is specific and sensitive, but time-consuming and laborious, and therefore it is not widely used in routine diagnostic services.

### Hemagglutination Inhibition Test

Many viruses bind to hemagglutinin molecules found at the surface of red blood cells of various animal species and this can cause aggregation of red cells in suitable conditions. Prevention of this aggregation, called hemagglutination inhibition, by specific antiviral antibodies in the patient's serum has been widely used for diagnostic purposes. The test, known as hemagglutination inhibition test, has important diagnostic and public health applications in certain infections, most notably in influenza where antibodies measured by this test show additional specificity compared to other tests and therefore provide more detailed information about the immunity and past infections of individuals. However, for the diagnosis of individual patients, the assay is no longer widely used and is replaced by more modern immunoassays.

In the test, a virus preparation with a predetermined hemagglutinating capacity is mixed with the serum sample and after proper incubation the residual hemagglutination capacity is measured. Both IgM and IgG antibodies are able to inhibit hemagglutination.

### Complement Fixation Test

The complement fixation test (CFT) is a classical laboratory diagnostic test, which is still used for determination of virus antibodies in patient sera or cerebrospinal fluid samples during an acute infection. The test mainly measures IgG antibodies.

The test is based on the capacity of complement, a group of heat-labile proteins present in the plasma of most warm-blooded animals to bind to antigen–antibody complexes. When the complexes are present on the surface of red blood cells, complement causes their lysis which can be visualized by a suitable experimental setup.

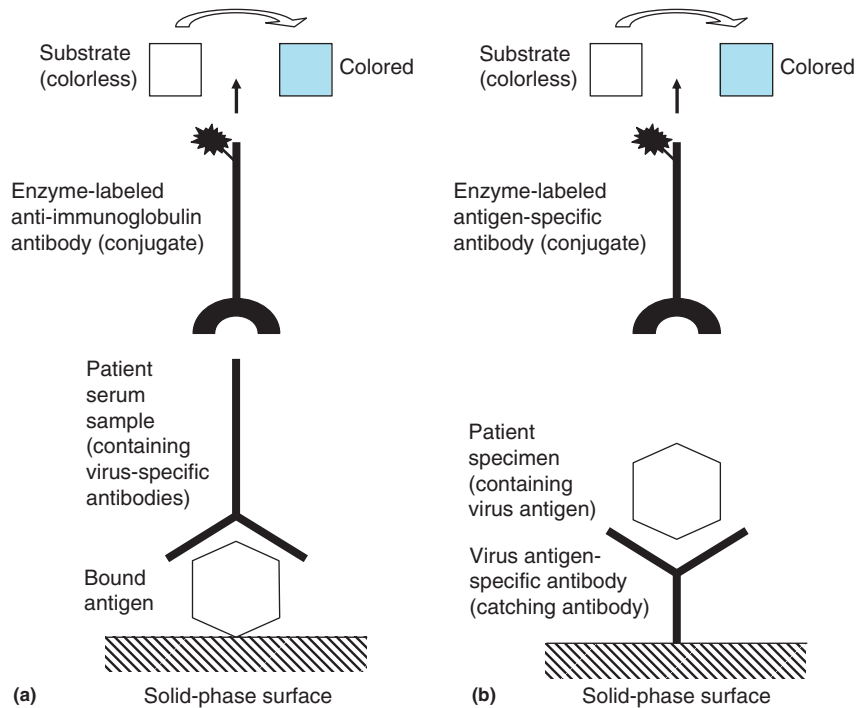
In the actual test, the complement in the patient's serum is first destroyed by heating; the serum is then mixed with appropriate viral antigen and after incubation; when the antigen–antibody complexes are formed, exogenous complement (usually from fresh guinea pig serum) is added. This complement then binds to the complexes and having been 'fixed,' it is then no longer able to cause lysis of added indicator red cells. Usually, sheep red cells coated with antish sheep red cell antibodies are used as indicator to measure the presence of any residual complement. The effect is measured by a suitable test protocol. Serial dilutions of the patient serum are used and the highest dilution where the serum can still prevent complement activity in the indicator system is taken as the CFT titer of the sample. The tests are usually carried out on microtiter plates and the results are observed by eye.

CFT is still used for diagnosis of acute virus infection. It measures certain types of antibodies which occur only during the acute phase of the infection. Therefore, CFT is not suitable for investigation of immune status. The assay procedure is quite complex, because the test is dependent on several biological variables, which have to be standardized by pretesting. The method is less sensitive than many other immunoassays. In addition, the method is very labor intensive and is not amenable to automation. The use of CFT in virus diagnostics is increasingly replaced by modern immunoassays.

### Immunoassays

In immunoassays, antibodies binding to specific immobilized antigens can directly be observed using bound antigens and proper indicators such as labeled anti-immunoglobulin antibodies. The antigens can be immobilized to plastic microtiter plates, glass slides, filter papers or any similar material. Different immunoassays are nowadays widely used to measure virus-specific IgM and IgG antibodies. The most recent formats of immunoassays make it possible to detect simultaneously both antigens and antibodies decreasing significantly the window period between infection and immune response. Numerous commercial kits with high specificity and sensitivity are available. Automation has made immunoassay techniques more rapid, accurate, and easier to perform.

In the basic format of solid-phase immunoassays, virus-infected cells, cell lysates, purified or semipurified, recombinant viral antigens or synthetic peptides are immobilized to a solid phase, usually plastic microtiter wells or glass slides. Patient's serum is incubated with the antigen and the bound antibody, after washing steps, is visualized using labeled anti-immunoglobulin antibodies ('conjugate') (Figure 2(a)). If the label used is an enzyme, the test is called enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) and the bound antibody is detected by an enzyme-dependent color reaction. If a fluorescent label is used, the method is called immunofluorescent test (IFT). The enzyme labels most commonly used are horseradish peroxidase (HRP) and alkaline phosphatase (AP). In HRP-EIA the color-forming system consists of ortho-phenyldiamine (OPD) as a chromogen and hydrogen peroxidase ( $H_2O_2$ ) as a substrate. If the HRP-conjugate is bound to antibody–antigen complexes, the colorless chromogen becomes yellow and color intensity is measured with a photometer at a wavelength of 490–492 nm. The intensity of the color is proportional to the amount of bound conjugate and to the amount of specific antibodies in a patient serum sample. If the serum contains no specific antibodies, the conjugate is not bound and no color reaction occurs. By using either anti-IgG or anti-IgM conjugates it is possible to determine separately immunoglobulin subclasses.



**Figure 2** Enzyme immunoassay. (a) Detection of virus-specific antibodies. (b) Detection of virus antigens.

The specificity and sensitivity of these immunoassays are generally high. The most common source of potential misinterpretation is false positive IgM reaction due to the presence of rheumatoid factor, itself IgM molecule, reacting with virus-specific IgG. The specificity can be improved by using an additional incubation step where IgM antibodies are first enriched ('captured') in the sample by using anti-IgM immunoglobulin. Alternatively IgG can be adsorbed or blocked by serum pretreatment.

Immunofluorescent tests were used in the past for measuring virus-specific antibodies, but are now replaced by EIA techniques. The principle of the method is similar to EIAs. In IFT, infected cells are placed on a glass slide and bound antibodies are detected by fluorescein-labeled anti-immunoglobulin antibodies. The glass slides are examined under a fluorescence microscope. The method is specific and sensitive, but quite labor intensive, and reading the test demands considerable experience.

### Immunoblotting

In some infections (e.g., that caused by human immunodeficiency virus (HIV)), antibodies against certain components of the virus are more informative than other less-specific antibodies and they are detected by immunoblotting assays. Different virus antigens, prepared by gel diffusion or other techniques, are absorbed as discrete bands on a solid strip of cellulose or similar material and the strip is incubated with the patient's serum. Antibodies present in the serum bind to specific antigens and are detected using an HRP-conjugate and nitroblue tetrazolium as the precipitating color chromogen. The color reaction is observed and compared to positive and negative control samples assayed on separate strips. Immunoblotting assays are commonly used to confirm highly sensitive EIA screening assay results for antibodies against HIV, hepatitis C virus (HCV) and human T-cell lymphoma virus (HTLV).

### Lateral-Flow and Latex Tests

A technique known as lateral-flow technology has also been used to identify antibodies or antigens. These tests involve application of serum or other samples directly on a strip of suitable material such as cellulose, where the antibodies are diffused laterally and eventually reach a site in the strip where appropriate antigen has been applied and chemically fixed. Specific antibodies become bound to the site while nonreacting antibodies diffuse out from the area. The presence of antibodies is visualized using labeled conjugates.

Although such tests are not quantitative, they are valuable for infections where the presence of specific antibodies is indicative, such as HIV infection. Performance of the test is often very simple and the result is available in a few minutes or a few hours, making such tests suitable for bed-side screening. In more advanced tests, several different antibodies can be detected by a single assay and the test conditions can be modified further so that antigens can also be detected. Many such tests have become commercially available in recent years.

For some applications, coated latex particles have replaced strips with fixed antigen as the solid phase. Binding of specific antibodies can be visualized with chromogenic or otherwise labeled indicator antibodies or a positive reaction can be detected by agglutination of the latex particles.

### Detection of Viral Antigens

The presence of viral antigens in clinical specimens, such as nasopharyngeal aspirates, fecal specimens, vesicle fluids, tissue specimens, as well as serum samples can be demonstrated by antigen detection assays.

In immunofluorescence tests, cells from a clinical specimen are fixed on a glass slide and viral antigens present in the cells are detected by fluorescein-labeled virus-specific antibodies. Less reader-dependent results can be obtained using enzyme or other immunoassays. Solubilized antigens in clinical specimens are first captured using specific monoclonal antibodies bound to a solid phase, and are then detected with virus-specific detector antibodies (Figure 2(b)). The functionality of monoclonal antibodies (MAbs) with high specific binding affinity is better preserved when labeled with a small molecule as compared to a bulky enzyme molecule. In EIA, biotinylated MAbs are used with streptavidin-enzyme conjugate. In time-resolved fluoroimmunoassay (TR-FIA), detector MAbs are labeled with an europium chelate.

Antigen detection methods are especially recommended in the case of virus reactivation, for example, for herpes simplex and varicella zoster virus diagnosis where the serological response can be very weak. Antigen detection assays are also widely used in respiratory tract infections like influenza and respiratory syncytial virus infections. A simple test for the demonstration of rotavirus and adenovirus antigens in children with gastroenteritis is also available.

### Nucleic Acid Detection Assays

Direct demonstration of viral nucleic acids in clinical samples has become the technique with the widest repertoire of diagnostic virus targets. Using the polymerase chain reaction (PCR) with specific primers, viral sequences can be rapidly multiplied and identified. These techniques have largely replaced classical virus isolation. They are rapid to perform and in many cases more sensitive than virus isolation or antigen detection methods making earlier diagnosis possible. They have proved particularly valuable for the diagnosis of emerging viruses and viruses that cannot be cultivated such as papillomaviruses, parvoviruses, hepatitis viruses, and rhinovirus species C. Semiquantitative and quantitative applications have been developed allowing monitoring of viral load during antiviral treatment. These tests cannot distinguish between viable and replication-incompetent virus, warranting caution in the interpretation of the results in certain cases. While sensitivity to cross-over contamination throughout the diagnostic pathway cannot be undermined, use of real-time PCR technology has diminished these problems in clinical laboratory settings.

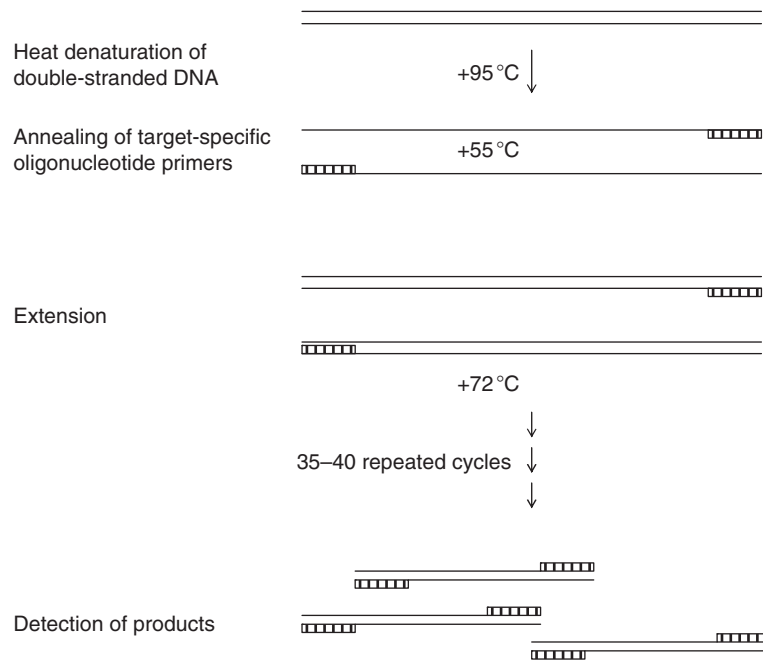
The specificity of these tests is based on the extent of pair-matching sequences between the viral nucleic acids and the primers. Extremely high sensitivity is typical for PCR methods; 1–10 copies of viral nucleic acid can be detected in about one to few hours. PCR methods are available for both RNA and DNA viruses. For RNA viruses viral nucleic acid has to be transcribed with reverse transcriptase (RT) enzyme to complementary DNA in a combined assay called RT-PCR.

Viral nucleic acid is extracted from the sample material and amplified in three successive steps. The double-stranded DNA is first heat-denatured and separated into single strands. The specific target fragment of DNA strand is then amplified (Figure 3) by pairs of target-specific oligonucleotide primers, each of which anneal to one strand of unfold double-stranded DNA. Each annealed primer acts as an origin for heat-stable polymerase enzyme and a complementary strand is synthesized via sequential addition of deoxyribonucleotides. In real-time PCR, annealing and extension steps are often combined to a single step at 60 °C. These cycles are repeated about 40 times, each cycle resulting in an exponentially increasing numbers of copies.

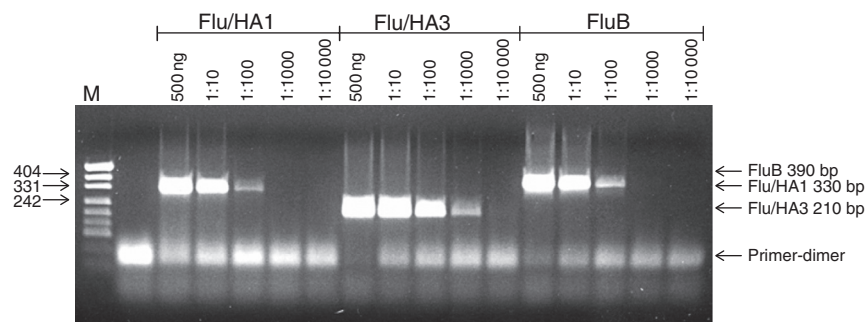
After the amplification is completed, the products can be detected by several methods. Agarose gel electrophoresis combined with ethidium bromide staining of the products is a classical method (Figure 4). The size of the amplified product is compared to control amplicons and other standards in the same gel. Various hybridization assays, based on labeled complementary oligonucleotides (probes), are also used to improve the sensitivity and specificity of the detection. For applications with a large number of targets, such as respiratory virus detection or papillomavirus typing, conventional PCR with post-PCR hybridization is still a popular solution. Products of highly multiplexed PCR assays can be identified by probe hybridization on microarrays using scanning fluorometer or on fluorescent microbeads using flow-cytometric bead counting.

The amplified fragments can also be sequenced giving additional information about the virus. Comparison of the sequences with known virus sequences allows identification of species, strains, or subtypes that may be important for public health or medical purposes. Sequencing after RT-PCR is also the current method-of-choice for investigating the emergence of antiviral drug resistance among HIV-infected patients.

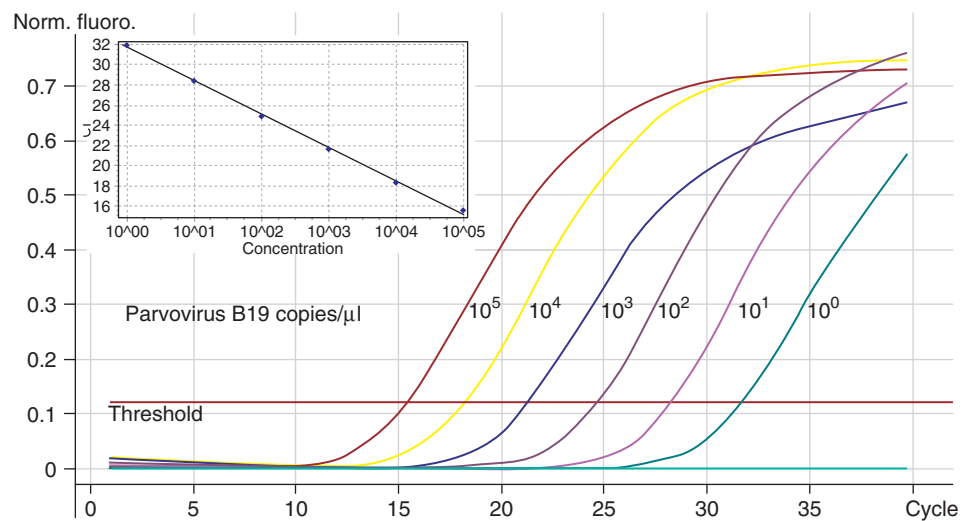
Real-time PCR instruments monitor accumulation of amplicons by measuring the fluorescence continuously in each cycle of the reaction. Fluorescence is generated by a dsDNA dye or a fluorescent probe system included into the reaction mix. The earlier the amplification product becomes detectable over the background, the higher is the amount of virus in the sample (Figure 5). A dsDNA dye (e.g. SYBR Green I) reacts with any dsDNA formed during amplification, but amplicon specificity can be confirmed by melting curve analysis. It also allows testing for more than one virus from the same sample (Figure 6). There are many



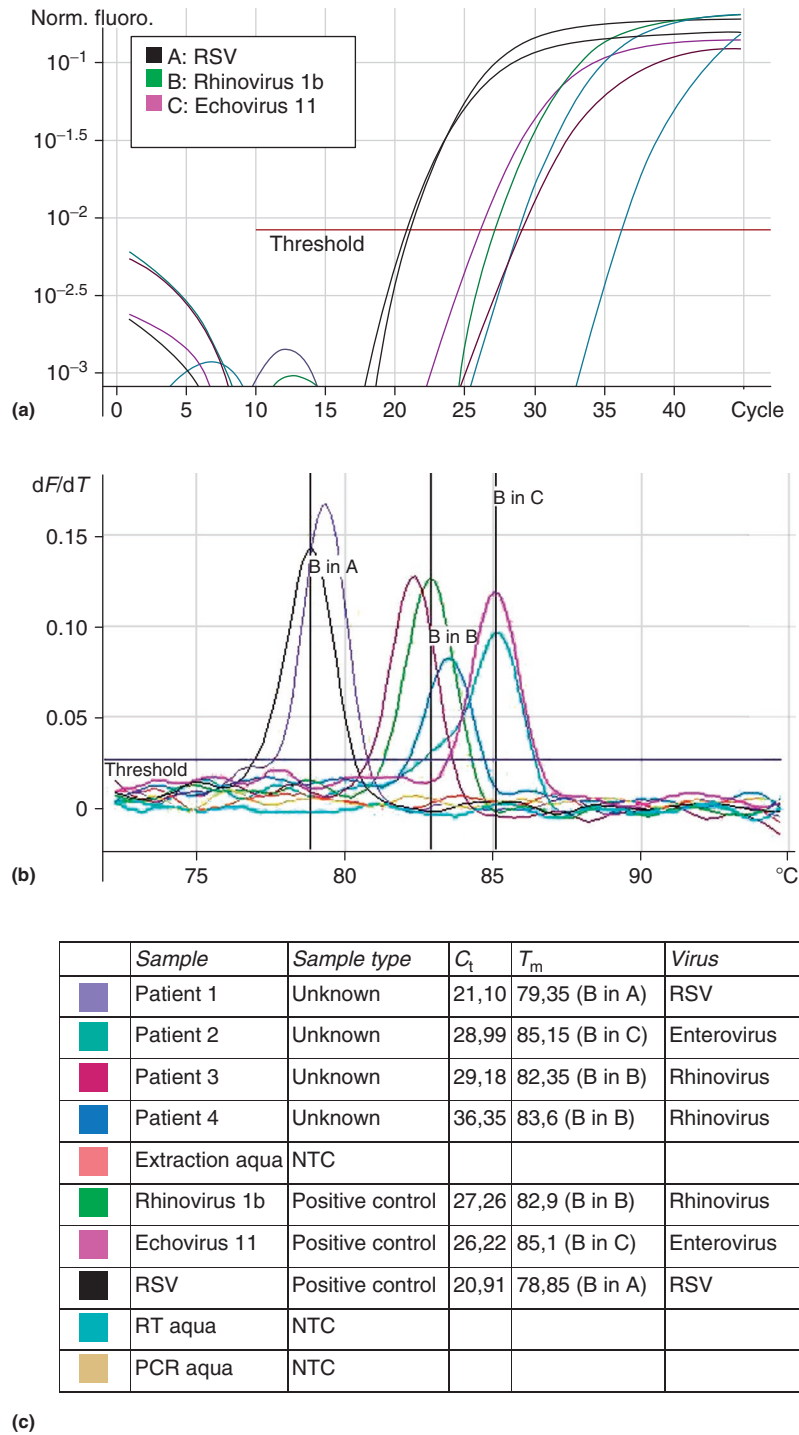
**Figure 3** Polymerase chain reaction.



**Figure 4** Detection of PCR products (amplicons) by an agarose gel electrophoresis after ethidium bromide staining.



**Figure 5** Quantitative real-time PCR with fluorescent-labeled probes for parvovirus B19.



**Figure 6** RT-PCR with real-time detection (a) and with melting curve analysis (b) for the detection of respiratory syncytial virus (RSV), rhinovirus, and enterovirus in respiratory secretions (c).  $C_t$  is a threshold cycle number,  $T_m$  is a melting temperature, and NTC is a nontemplate control. Unpublished results by Waris M, Tevaluoto T, and Österback R.

alternative probe chemistries for real-time PCR, the most common including dual label probes. Since probes are usually more selective for their target sequences than primers, probe based real-time PCR methods offer highest specificities with fastest reaction times. Multichannel instruments allow the use of probes with different excitation and emission spectra for the simultaneous detection of several different analytes in a single tube.

The PCR assays are extremely sensitive and can therefore be influenced by inhibitors of the polymerase enzyme that are sometimes present in clinical samples. Internal controls can be included into reaction mixtures. Nucleases present in samples or in

reagents can also cause false negative results by degrading viral nucleic acids. Furthermore, amplicons may also cause product carryover and false positive results. Extremely high care has to be applied in handling the clinical specimens, the reagents, as well as the reaction products.

The key to a successful PCR method is the careful design of the primers and probes. They have to be specific for the target and follow certain structural rules to produce efficient amplification. The high specificity of the PCR, especially probe-based real-time PCR, makes the technique sensitive for mutations, sense or non-sense, in the target sequences of highly variable viruses. That must be taken into the consideration not only in design of the assays, but also in continuous evaluation of them for changes in sensitivity caused by new mutations affecting critical primer or probe binding sites.

One of the great advantages of the PCR technology is its potential to detect new emerging viruses. By using primers from related viruses or so-called generic primers important information regarding the new virus can be obtained for further development of more specific tests. A recent example is the Middle-East respiratory syndrome (MERS) coronavirus, for which specific diagnostic tests became available soon after the taxonomic position of the virus became known. The technology also allows safe handling and transport of virus samples, since extraction buffers added to the samples inactivate virus infectivity.

### **Point-of-care Tests**

POC tests are becoming increasingly common in clinical practice. In proper use, they offer a cost-efficient diagnostic guidance for quick clinical decision making. Their performance is rarely as good as that of corresponding laboratory tests; use of them should be quality-controlled, and a back-up should be arranged with a virology lab. Most of them are based on easy-to-use lateral-flow or latex particle technology and are able to give the result in a few minutes. POC tests are nowadays available for antibody screening of an increasing number of virus infections (HIV, hepatitis C virus (HCV), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein–Barr virus (EBV)).

With the breakthrough of nucleic acid testing, antigen detection methods are becoming obsolete in high-end virus laboratories, but their development have continued for POC testing. Most commercially available tests for antigen detection are lateral flow immunochromatographic assays, and they are typically targeting influenza viruses, respiratory syncytial virus, adenoviruses, rotavirus, norovirus and hepatitis B virus. Multianalyte testing for 8 common respiratory virus antigens is possible with a commercial multianalyte respiratory infection POC test platform including a small bench-top instrument suitable for polyclinical use. Also compact all-in-one PCR systems for the detection of respiratory pathogens are now available.

### **Future Perspectives**

Driven by public health, scientific and commercial interests, new diagnostic tests for the laboratory diagnosis of viral infections are continuously being developed. The development of new molecular detection methods continues on two lines. On the one hand, it includes compact, fully integrated automates requiring minimal training to run and suitable for POC use. On the other hand, separate instruments for specimen handling, nucleic acid extraction, PCR set-up, and amplification are integrated into high throughput flow systems, which allow efficient use of both commercial and laboratory designed assays.

Use of multianalyte methods is becoming a practical reality and they might significantly change diagnostics of infectious diseases in future. Multiplex PCR in a single tube or miniature format still have the challenge of reaching the sensitivity of simpler PCR targeting only one or few analytes. Microarray technology for PCR product detection competes with sequencing, but still provide a rapid alternative for identification of a large number of virus types. Next generation sequencing technology will be the primary way of identification of new or emerging viruses. Simple microarrays, readable without bioinformatics, would reduce the cost of serological screening and allow the use of control measures to improved quality of the results. Outside the specific virus diagnostics, measures of virus induced interferon response is an area of potential development.

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