Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

the labeled Padlock amplicons to oligonucleotide arrays (**Figure 10(b)**) or as binding region for a TaqMan probe or a molecular beacon in quantitative real-time PCR (**Figure 10(c)**).

RNase Protection Assay

These tests offer a simple and cheap method to quantify RNA without PCR. They are especially useful when several RNAs have to be quantified simultaneously. Discrete-sized complementary RNAs are labeled, for example, by radioactive isotopes, and are hybridized in solution with the target RNAs to yield labeled dsRNAs. After RNase digestion of the unhybridized probe and target RNAs, the labeled dsRNAs are separated electrophoretically in a denaturing polyacrylamide gel and are visualized and quantified according to the label used.

See also: Diagnostic Techniques: Microarrays; Electron Microscopy of Viruses.

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Diagnostic Techniques: Serological and Molecular Approaches

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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. Serological methods are used for measuring the antibody response while the presence of virus can be demonstrated by cultivation or demonstration of specific antigens or gene sequences. For the latter, molecular diagnostic methods have become more and more widely applied.

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. 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.

The presence of infectious virus or viral structural components can be investigated directly from various clinical specimens either by virus isolation, nucleic acid detection assays, or antigen detection assays. 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.

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 7–10 days 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 acute infection the specific avidity of IgG antibodies is usually low but it increases
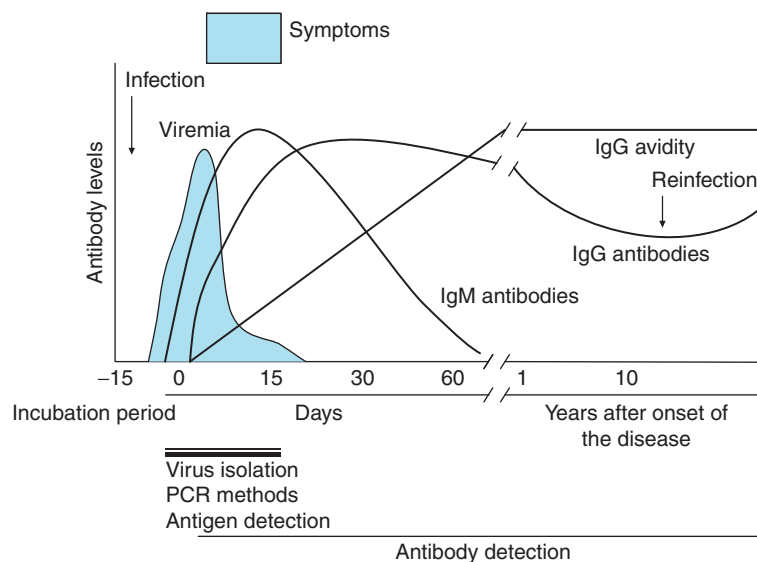
Figure 1 The course of virus infection. The shedding of infectious virus after incubation period and typical antibody response. Recommended diagnostic laboratory methods have been marked.

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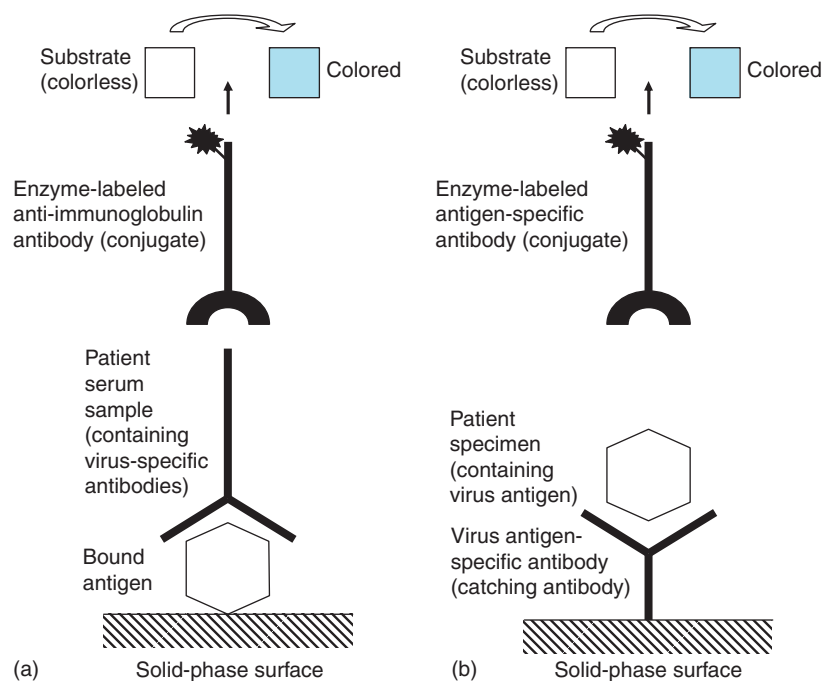
Figure 2 Enzyme immunoassay. (a) Detection of virus-specific antibodies. (b) Detection of virus antigens.

The specificity and sensitivity of these immunoassays are high. The sensitivity can be improved further, by using an additional incubation step where IgM antibodies are first enriched ('captured') in the sample by using anti-IgM immunoglobulin. Modifications to improve assay specificity by various methods of antigen handling and by using monoclonal antibodies or synthetic peptides have been developed.

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 immuno-deficiency 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.

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.

Point-of-Care Tests

Point-of-care tests (POC tests) are becoming increasingly common in clinical practice. 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)). Some authorities still question the validity of POC tests for clinical use although there is considerable evidence that many of the commercially available kits give reliable results.

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. More reliable results can be obtained using enzyme immunoassay or time-resolved fluoroimmunoassay (TR-FIA). Europium-labeled mono-clonal antibodies can be used as a conjugate. Solubilized antigens in clinical specimens are first captured using specific monoclonal antibodies bound to a solid phase, and are then detected with enzyme- or europium-labeled virus-specific antibodies (**Figure 2(b)**).

Antigen detection methods are especially recommended in the case of virus reactivation, for example, for herpes simplex and varicella zoster virus diagnosis where the sero-logical 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 is an increasingly used technique for virus diagnosis. Using the polymerase chain reaction (PCR) with specific

primers, viral sequences can be rapidly multiplied and identified. These techniques are largely replacing 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 viruses that cannot be cultivated such as papillomaviruses, parvoviruses, and hepatitis viruses. 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. Also, sensitivity to cross-over contamination in the laboratory has caused some 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 a 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 (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 hybridize to one strand of double-stranded DNA. The hybridized primers act as an origin for heat-stable polymerase enzyme and a complementary strand is synthesized via sequential addition of deoxyribonucleotides. After annealing of the primers, extension of the DNA fragment will start. These cycles are repeated 35–40 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
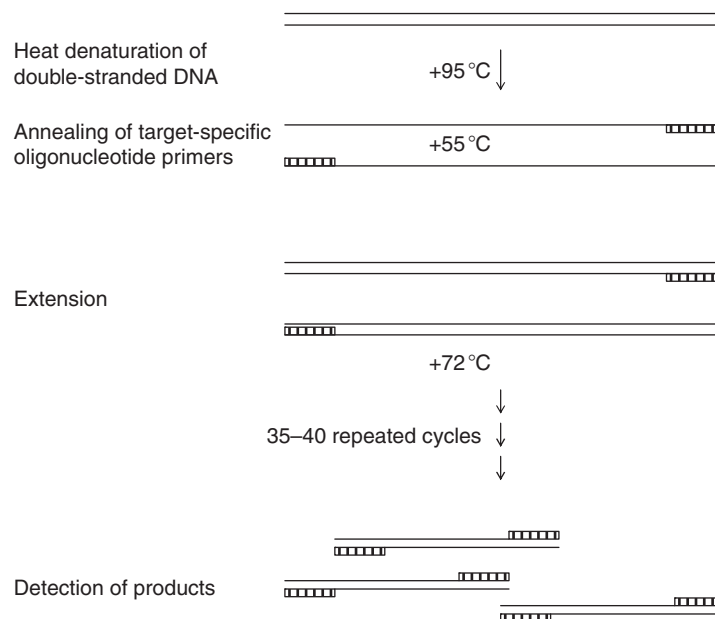
Figure 3 Polymerase chain reaction.
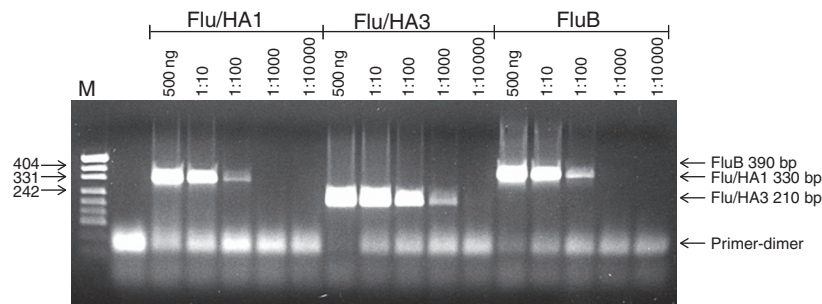
Figure 4 Detection of PCR products (amplicons) by an agarose gel electrophoresis after ethidium bromide staining.

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.

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. The earlier the amplification product becomes detectable over the background, the higher is the amount of virus in the sample (**Figure 5**). One application, based on the use of melting temperatures, allows simultaneous detection and analysis of several different nucleic acids. It also allows testing for more than one virus from the same sample (**Figure 6**).

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.
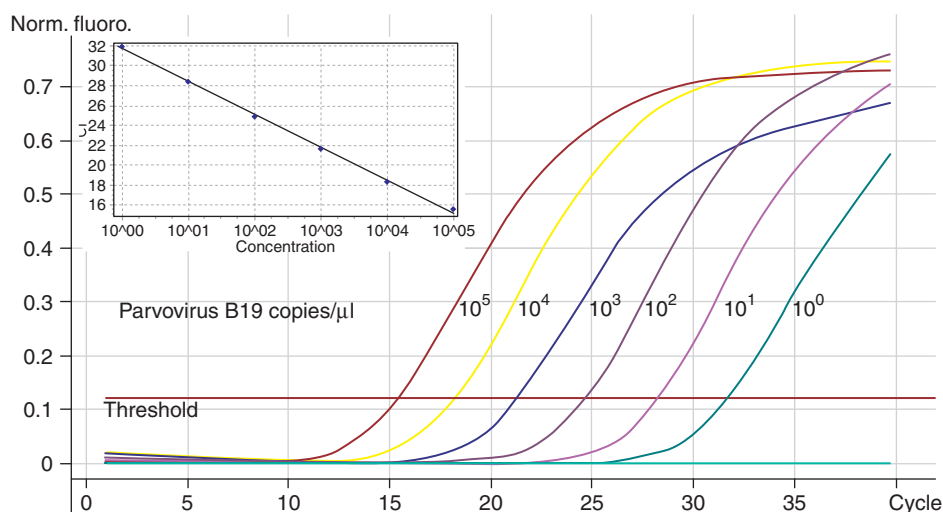
One of the great advantages of the PCR technology is its potential to detect new emerging viruses. By using primers from related viruses or so-called generic primers important information regarding the new virus can be obtained for further development of more specific tests. A good example is the severe acute respiratory syndrome (SARS) virus, 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.
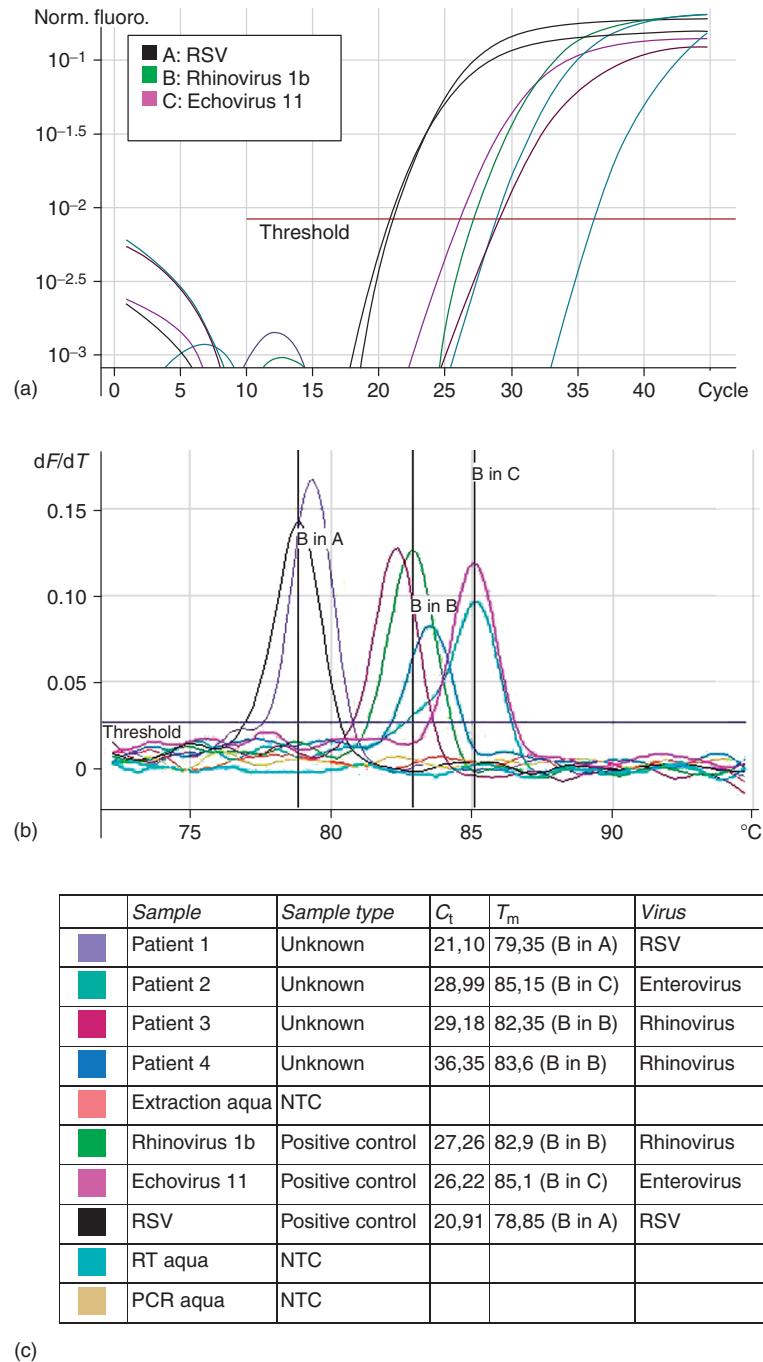
Future Perspectives

Driven by public health, scientific and commercial interests, new diagnostic tests for the laboratory diagnosis of viral infections are continuously being developed. The main area for development will probably be new molecular detection methods, where automation will provide rapid, well-standardized, and easy-to-use technology.

Use of multianalyte methods is becoming a practical reality and they might significantly change diagnostics of infectious diseases in future. They provide an opportunity to screen simultaneously for a wide range of viruses increasing the rapidity of the diagnostic procedure. A single microarray test (microchip) can contain thousands of virus-specific oligonucleotide probes spotted on a glass slide. Several kit applications for detecting viral nucleic acids and antigens or virus-specific antibodies already exist. Microarrays based on random PCR amplification can be used to detect a variety of viruses belonging to different families. Screening of some other infection markers can be also included in the same test format. Microarrays are not widely used for clinical purposes because of limited sensitivity and the difficulties of developing analytical instruments suitable for diagnostic laboratories. Another line of

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

(a)

(b)

	Sample	Sample type	C_t	T_m	Virus
	Patient 1	Unknown	21,10	79,35 (B in A)	RSV
	Patient 2	Unknown	28,99	85,15 (B in C)	Enterovirus
	Patient 3	Unknown	29,18	82,35 (B in B)	Rhinovirus
	Patient 4	Unknown	36,35	83,6 (B in B)	Rhinovirus
	Extraction aqua	NTC			
	Rhinovirus 1b	Positive control	27,26	82,9 (B in B)	Rhinovirus
	Echovirus 11	Positive control	26,22	85,1 (B in C)	Enterovirus
	RSV	Positive control	20,91	78,85 (B in A)	RSV
	RT aqua	NTC			
	PCR aqua	NTC			

(c)

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.

development is the increasing number of POC tests, which may form an important part of future diagnostic testing of infectious diseases.

See also: Antigenicity and Immunogenicity of Viral Proteins; Diagnostic Techniques: Microarrays; Immune Response to viruses: Antibody-Mediated Immunity.

Further Reading

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Dicistroviruses

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Glossary

Cohort A group of similar individuals.

Dipteran A member of the insect order Diptera: true flies.

Hemipteran A member of the insect order Hemiptera: true bugs (including aphids).

Hymenopteran A member of the insect order Hymenoptera: wasps and bees.

Intergenic region Region between the two open reading frames in the dicistrovirus genome.

Lepidopteran A member of the insect order Lepidoptera: moths and butterflies.

Orthopteran A member of the insect order Orthoptera: crickets and grasshoppers.

Penaeid A shrimp from the family Penaeidae.

Picorna-like Viruses that are ostensibly like members of the *Picornaviridae*; but the term is generally used to refer to any small (*c.* 30 nm in diameter) icosahedral viruses with single-stranded RNA genomes.

Polyprotein A protein that is cleaved after synthesis to produce a number of smaller functional proteins.

Vertical transmission Transmission of virus directly from an infected mother to her offspring.

VPg A virally encoded protein covalently linked to the 5′ end of the viral genome.

Introduction

After the early 1960s, it became apparent that invertebrates, as well as vertebrates and plants, played host to a number of small (<40 nm in diameter) icosahedral viruses with RNA genomes. The initial descriptions of many of these viruses involved little more than their physical and biochemical characteristics such as diameter, density, and S-value. By the 1970s, many new invertebrate small RNA viruses were being isolated and described and even minimal characterization made it clear that new families of viruses were emerging from this assemblage of small RNA-containing viruses of invertebrates, along with apparent members of existing virus families. Two major families of viruses recognized in the late 1970s and early 1980s were the *Tetraviridae* and *Nodaviridae*. Any of the other viruses were simply considered to be 'invertebrate picornaviruses' or 'small RNA viruses of insects'.

The properties of many of the yet-unclassified viruses were found to be very similar to those of the mammalian picornaviruses. In particular, the size of the virions (*c.* 30 nm), the composition of the capsids (three major proteins of around 30 kDa), and single-stranded, positive-sense RNA genomes all suggested these were invertebrate picornaviruses and this was very much the prevalent feeling – until 1998. At this point, the first full genome sequence of one such invertebrate virus, drosophila C virus (DCV), was published and surprisingly revealed a genome organization strikingly different from the picornaviruses, and indeed quite different from any other viruses known at that time. During the next several years, the genomes of a number of insect small RNA-containing viruses were sequenced and it became clear that two organizational paradigms existed. The first group became the *Dicistroviridae* while the second has become the (currently) unassigned genus *Iflavirus*.

Taxonomy and Classification

The family *Dicistroviridae* currently comprises 12 species, most of them in the only genus recognized so far, *Cripavirus* (**Table 1**). There are a number of other potential candidates for the family but these have yet to be accepted as species by the International Committee on Taxonomy of Viruses. For the purposes of this article, we will limit our discussion to only those species shown in **Table 1**.