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Handbook of Clinical Neurology, Vol. 123 (3rd series)
Neurovirology
A.C. Tselis and J. Booss, Editors

Chapter 5

Laboratory diagnosis of viral infection

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INTRODUCTION

Since the last *Handbook of Clinical Neurology* volume on this topic, viral diagnosis has made tremendous strides, moving from the margin to the mainstream of clinical care. This change has been driven by many factors, but most importantly by effective antiviral therapies. For many years, conventional virus isolation was the mainstay of viral diagnosis since it was sensitive and "open-minded." However, cell culture is a specialized technique that requires highly skilled personnel. In addition, growth in conventional cell culture entails an inherent delay that limits its clinical impact. Although rapid culture and viral antigen methods detect fewer pathogens and are less sensitive than conventional culture, both require less expertise and have greatly reduced time to result. In the previous edition of this text, nucleic acid hybridization was highlighted; however it was more esoteric and expensive than other methods, yet not more sensitive and thus had limited clinical application.

Polymerase chain reaction (PCR) has ushered in a new era in virology, especially in the diagnosis of neurologic diseases. Molecular amplification methods, including, but not limited to, PCR, are both rapid and highly sensitive, can detect viruses not amenable to routine culture, can be automated, and can be quantitative. As the variety of available test methods and commercial products proliferate, the challenge for clinicians and laboratories is selecting which tests to utilize in which clinical scenarios, and understanding how to interpret the results. Each test modality has advantages and limitations (Table 5.1), which will be discussed in this chapter, with special attention to neurologic disease. For a more detailed description of test methods, readers are referred to textbooks on viral diagnosis (Specter et al., 2009; Jerome, 2010; Tang and Crowe, 2011).

GENERAL PRINCIPLES OF LABORATORY VIRAL DIAGNOSIS

Viral diagnostic testing is undertaken either to document prior infection or to assist in the diagnosis of an acute illness. In this chapter, we will focus on disease diagnosis. In an idealized viral infection (Fig. 5.1), viral replication, clinical symptoms, the appearance of immunoglobulin M (IgM) and IgG antibodies, the elimination of virus, and resolution of disease occur in a predictable progression. When disease is a consequence of viral replication and host cell lysis, diagnostic efforts should be focused on detection of virus, and samples should be collected as early as possible in illness when virus titers are maximal. Alternatively, some disease processes are mediated by the host response to viral infections, and detection of antibody is key. Viruses associated with immune-mediated diseases include Epstein–Barr virus (EBV), hepatitis B virus (HBV), and parvovirus B19. Unfortunately, there are important deviations from the scenario of acute infection progressing to viral clearance, such as viral persistence, latency, reactivation, and late disease. Thus the pattern for a particular virus, as well as the immune competence of the host, should be considered when ordering tests and interpreting the results.

VIRAL DIAGNOSTIC METHODS

Conventional cell culture

Viruses require living cells in order to replicate. Early work in virology utilized primates, embryonated eggs,

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Table 5.1

Overview of viral diagnostic methods: advantages and limitations

Technique		Assay time	Advantages	Limitations
Viral isolation	Conventional culture	1–21 days	Allows isolation of many viruses; can detect unexpected or novel viruses; more sensitive than antigen detection	Requires expertise to interpret CPE and maintain cell cultures; > 1 mL of CSF required for comprehensive culture; some viruses do not grow in routine cultures; biosafety concerns for zoonotic and emerging viruses
	Rapid culture	1–5 days	Most results in 1–2 days; requires less training to interpret IF staining than CPE; use of "mixed cell" cultures allows detection of multiple viruses in a single vial	Requires cell culture and IF expertise; detects only targeted viruses; less sensitive than conventional culture
Antibody detection	ELISA, EIA, CLIA, IF, IC, IB, IgG avidity testing	<30 min–24 hours	Can document primary, recent, and past infections, and carrier states; can be automated; some tests can be done at point of care; fourth-generation HIV tests combine antibody and antigen detection in one reaction	Cross-reactivity between similar viruses is common (e.g., arboviruses); diagnosis often retrospective; IgM assays have moderately high false-positive rates; immunocompromised hosts may not make antibody
Electron microscopy	Thin section	3 days	Allows visualization of virus particles; detection of unexpected pathogens and discovery of new viruses	Expensive; requires high viral burden and expertise in viral recognition; labor-intensive
	Negative stain	1 hour	Allows rapid visualization of virus particles in vesicle fluid, respiratory secretions, urine, or stool; detection of unexpected pathogens and discovery of new viruses	Expensive and labor-intensive; expertise is limited
Antigen detection	IF	1–2 hours	Can be done "on demand" as samples arrive in the laboratory; reagents available for eight respiratory and four herpesviruses; can assess sample quality	Requires substantial expertise for accurate results; manual and labor-intensive; requires an adequate number of target cells for valid results
	ELISA / CLIA	< 2 hours	Can be automated; requires less skill than IF	Limited test menu
	Membrane EIA	< 30 min	Requires no equipment; reagent additions and wash steps require lab-based testing	Largely supplanted by IC tests
	IC	< 30 min	Requires no equipment and little expertise; simply add sample and set timer; approved for use at "point of care"	Less sensitive than other methods; limited test menu

NAAT	General comments		Most sensitive method; detects viruses that do not grow in culture; more rapid than culture; safer than culture since pathogens are inactivated and disrupted before testing; potential for automation and quantification	Requires specialized equipment and expertise; results variable across laboratories; inhibitors can prevent amplification; cross-contamination leads to false positives; can detect clinically irrelevant viruses; genetic variability can lead to false-negative results; few FDA-approved assays
	Conventional PCR	5–9 hours	Uses inexpensive conventional thermocyclers; less affected by genome variability and more amenable to multiplex testing than real-time assays	Prone to carryover contamination from amplified products since tube is opened after amplification; slower than real-time methods; ethidium bomide used for amplicon detection is toxic
	Real-time PCR	1–5 hours	Faster, less prone to cross-contamination, readily quantified; lab-developed assays can be readily updated; more commercial kits becoming available, including walk-away tests	More prone to falsely negative or low values due to genetic variations in viral strains; lack of standardization; values obtained in different laboratories can vary by 3 log10; limited capacity to multiplex
	Other NAAT (e.g. bDNA, TMA, NASBA)	3–8 hours	Alternate methods have advantages in some situations; bDNA less affected by genome variability, less prone to contamination, and more reproducible than PCR	More limited test menus

bDNA, branched DNA (assay); CLIA, chemiluminescent immunoassay; CPE, cytopathic effect; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HIV, human immunodeficiency virus; IB, immunoblot; IC, immunochromatography; IF, immunofluorescent assay; IgM, immunoglobulin M; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; TMA, transcription-mediated amplification.

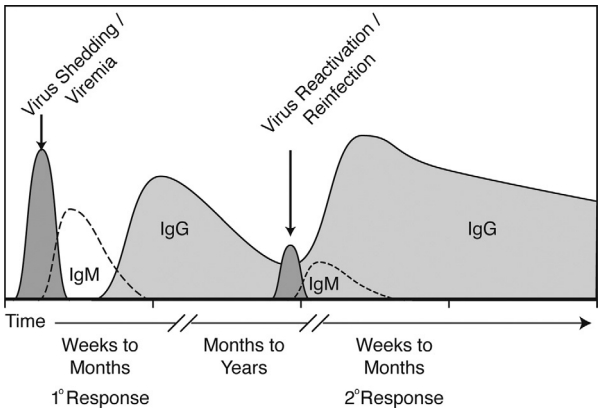

Fig. 5.1. Typical viral infection and immune response. With viral reactivation or reinfection, immunoglobulin (Ig) M response may or may not be detected.

suckling mice, and other susceptible animal hosts. The recognition that viruses could be propagated in cultured human and animal cells led to the Nobel prize for Enders, Weller, and Robbins, and isolation in cell culture became the gold standard for viral diagnosis (Mortimer, 2009). A variety of different cell lines are needed to culture a broad spectrum of clinically significant viruses (Landry, 2009a). In the clinical laboratory, three basic categories of cell lines are used: primary, diploid, and continuous or heteroploid (Table 5.2). Most laboratories performing conventional viral culture will inoculate a clinical specimen into two or three different cell lines depending on the viruses sought (Leland and Ginocchio, 2007). Cultures are incubated and monitored for 1–3 weeks for the appearance of characteristic viral cytopathic effects (CPE) (Fig. 5.2A). Time to detection of CPE in conventional culture is determined by the virus inoculum, the growth pattern of the specific virus, the cell line(s) inoculated, whether the culture is rotated, the temperature of incubation, and the frequency of examination. If CPE is seen, the virus isolate is identified most commonly by immunofluorescence (IF) staining of the cultured cells. In the absence of CPE, hemadsorption (applying a weak solution of guinea red cells to the cell monolayer) can be used to screen for the presence of hemagglutinin proteins expressed on the surface of the cells infected with influenza or parainfluenza viruses. An acid lability test can be used to differentiate between rhinoviruses (acid-labile) and enteroviruses (acid-stable).

Advantages of conventional viral culture include the ability to detect many viruses, to obtain a viral isolate for

Table 5.2

Culture methods and time to detection

		Growth in conventional cell cultures*				
Group	Virus	RhMK	MRC-5	A549	Mean days to detect (range)	Rapid culture available†
Respiratory	Influenza A	+++	–	–	3 (1–7)	R-mix
	Influenza B	+++	–	–	3 (1–7)	R-mix
	RSV	++	+	++	6 (2–14)	R-mix
	Parainfluenza 1–3	+++	–	+	6 (1–12)	R-mix
	Adenovirus	+	++	+++	6 (1–14)	R-mix
	Rhinovirus	–	+/–	–	6 (1–14)	No
	Enterovirus	++	+	+	4 (1–10)	Super E-mix
	Human metapneumovirus	–	–	–	NA	R-mix
	Coronavirus (OC43, 229E, NL63)	–	–	–	NA	No
Herpesviruses	CMV	–	+++	–	10 (1–21)	MRC-5
	HSV 1, 2	–	+++	+++	2 (1–7)	H&V mix or MRC-5
	VZV	+	++	++	6 (3–14)	H&V mix or MRC-5
	EBV	–	–	–	NA	No
	HHV-6	–	–	–	NA	No
Gastrointestinal	Rotavirus	–	–	–	NA	No
	Norovirus	–	–	–	NA	No
	Enteric adenovirus	–	–	–	NA	No

*RhMK, primary rhesus monkey kidney cells; MRC-5, human diploid fibroblasts; A549, human carcinoma continuous cell line.
†R-mix, mixture of Mink lung or MDCK and A549 in one vial; Super E-mix, mixture of BGMK-hDAF and A549; H&V mix, CV-1 and MRC-5.
CMV, cytomegalovirus; EBV, Epstein–Barr virus; HHV-6, human herpesvirus-6; HSV, herpes simplex virus; MDCK, Madin-Darby canine kidney; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

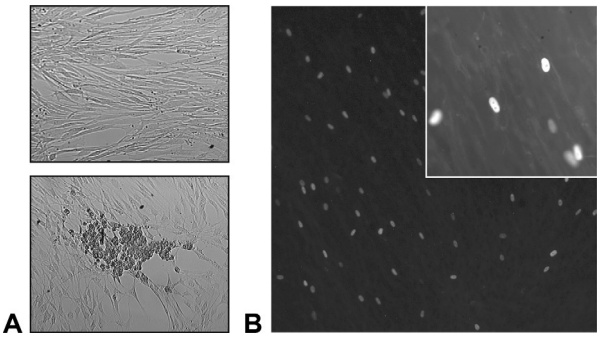

Fig. 5.2. Conventional and rapid cytomegalovirus (CMV) cultures.

A, Uninfected conventional MRC-5 cell culture (upper panel) and MRC-5 cells demonstrating CMV cytopathic effects 10 days after inoculation (lower panel).

B, Rapid shell vial culture (MRC-5 cells) 24 hours after inoculation. After fixation in acetone, the cell monolayer was stained with a monoclonal antibody to CMV immediate early antigen (100× magnification). Strong nuclear staining indicates CMV infection (inset, 400× magnification).

further testing, e.g., herpes simplex virus (HSV) antiviral resistance testing, and to identify new pathogens that have previously been unrecognized. The recently recognized human metapneumovirus (hMPV) and coronavirus NL63 were initially isolated using viral culture, but could not be identified with existing reagents (Van den Hoogen et al., 2001; van der Hoek et al., 2004).

Unfortunately, identification of viruses by conventional culture can take several weeks due to slow growth of some viruses and low viral inocula. Accurate interpretation of CPE requires a technically skilled and experienced laboratory staff that may not be available. When the volume of sample for testing is limited, for example, cerebrospinal fluid (CSF), fewer cell types can be inoculated and the yield of conventional culture is reduced (Landry et al., 2003). In addition CSF contaminated with blood may be toxic for cultured cells, some viruses cannot be grown using common cell lines, and culture for some viruses is restricted to higher biosafety-level facilities. Indeed, laboratories should be notified promptly when there is the possibility of recovering biosafety level 3 or 4 viruses, such as some arbovirus and zoonotic viruses (http://www.cdc.gov/biosafety/publications/), and alternative tests should be selected.

Rapid cultures

When a limited number of specific viruses are being considered in the differential diagnosis, a more targeted approach can be used, such as rapid culture using shell vials.

Shell vials, so named because of their resemblance to shotgun shells, are small vials that, at their base, contain a coverslip coated with a cell monolayer. After sample inoculation, the vials are centrifuged to increase sensitivity, leading to the synonym rapid centrifugation cultures. At predetermined time points after inoculation, the coverslips are removed from the shell vials, and the cell monolayers are fixed with acetone and subjected to IF staining with monoclonal antibodies (MAbs) for specific viruses (Fig. 5.2B). Shell vial cultures are most commonly examined after only 1–2 days of incubation and before the onset of CPE (Gleaves et al., 1984). In some cases, shell vials may be incubated longer and assessed for CPE.

Currently, many rapid culture systems use "mixed cells," instead of a single cell line. Mixed cells support the growth of several viruses that cause similar clinical syndromes, but involve the inoculation of only one cell mixture, rather than multiple vials containing different cell lines. For example, R-mix cells incorporate both mink lung and A549 cells into a single vial (Huang and Turchek, 2000). At 1–2 days postinoculation, the cultures are stained with a pool of MAbs to respiratory syncytial virus (RSV), influenza types A and B, parainfluenza types 1, 2, and 3, adenovirus, and, more recently, hMPV, before CPE is apparent (Fong et al., 2000; Weinberg et al., 2004). Neither cell line alone would allow for the recovery of all viruses. Mixed cells are also available for isolation of enteroviruses (Super E-mix) and for HSV-1, HSV-2, and varicella-zoster virus (VZV) (H&V Mix) (Huang et al., 2002a, b).

Genetically modified cell lines have been developed by transfecting a viral receptor into a cell line to enhance cell susceptibility to virus infection (e.g., Super E-mix cells), or by introducing genetic elements that, upon viral infection, will trigger production of a measurable enzyme and visible staining of infected cells (enzyme-linked inducible system or ELVIS) (Crist et al., 2004).

Rapid culture methods do not detect as wide a variety of viruses as conventional culture. However, compared to conventional culture, rapid cultures provide results in a more clinically relevant timeframe and can be implemented by less skilled personnel (Barenfanger et al., 2001). Thus, due to the combination of relative speed, sensitivity compared to antigen tests, and ease of use, rapid culture methods continue to find a niche in many viral diagnostic laboratories.

Serology

The presence of antibodies indicates past exposure to a virus, receipt of a vaccine, cross-reaction to a highly related virus, or even passive transfer of antibody if blood products or immunoglobulins have been administered. IgM antibodies are produced early after infection and are transient; thus the presence of antivirus IgM is considered synonymous with a current or recent

infection (James, 1990). Seroconversion from negative to positive IgG in an appropriate timeframe is the strongest evidence of a recent viral infection. In the proper clinical context, a fourfold rise in serum antibody titer is also considered diagnostic; however, few clinical laboratories still perform antibody titers (i.e., serial dilution of a serum sample and testing each dilution to determine the endpoint titer). Low-avidity IgG antibodies suggest a recent infection; thus avidity testing has been used during pregnancy to estimate whether cytomegalovirus (CMV) and rubella IgG reflects a recent primary infection (Hofmann and Liebert, 2005; Lazzarotto et al., 2008).

CSF can be tested for intrathecal production of antiviral antibodies as a marker of central nervous system (CNS) infection. For arboviruses, detection of IgM antibodies in CSF is preferred to molecular tests for viral nucleic acid, although use of both tests is necessary for maximum sensitivity (Prince et al., 2009). Detection of virus-specific IgG in CSF requires samples collected later in the course of infection than for IgM and exclusion of passive transfer of IgG from blood into CSF. IgG molecules more easily diffuse across the blood–brain barrier, and their presence in the CSF may simply reflect high serum antibody levels. Thus paired CSF and serum samples must be tested for antibody to the suspected pathogen, as well as to a control virus pathogen (Andiman, 1991). While commonly used for diagnosis of HSV encephalitis in the past (Aurelius et al., 1993), most CSF antiviral IgG testing has been replaced by IgM testing or by PCR.

Traditional serologic methods

The neutralization test measures the ability of the patient's antibody to neutralize infectivity and protect cells from infection, so it is considered a gold standard for the assessment of protective antibody. It is not used for routine diagnosis but is confined to specialty or public health laboratories for special indications, such as differentiating among highly related arboviruses and serotyping enteroviruses (Kuno, 2003; Malan et al., 2003). In this method, a challenge dose of infectious virus is mixed with serial dilutions of patient serum. After a 1-hour incubation, the mixture is inoculated on to cell culture monolayers. Most often, the monolayer is overlayed with a semisolid medium to facilitate the production of virus-infected foci or plaques. After a defined incubation period the monolayers are fixed and stained, and the virus-induced plaques are counted. The endpoint is the dilution of the patient's serum that reduces plaque formation by 90%. Thus this assay is also called a plaque reduction neutralization test (PRNT).

For many years complement fixation was widely used in clinical virology. While inexpensive, it is manual, requires careful in-house standardization and quality control, and is less sensitive than current methods. It is currently confined to public health laboratories when other methods are not available.

Hemagglutination inhibition was once widely used for viruses with surface proteins that agglutinate red blood cells. It is still used in public health or research laboratories to assess antibody response to specific strains of influenza viruses, especially after vaccination.

IF assays can be used for the detection of IgM or IgG, and commercial kits are available for EBV and arboviruses (Malan et al., 2003). Virus-infected cultured cells, mixed with uninfected cells, are affixed to glass microscope slides, and dilutions of patient serum are added. After an incubation period and wash step, bound antibody is detected by addition of antihuman antibody conjugated to fluorescein. IF is an excellent test when done well, but it requires a fluorescence microscope and extensive training and judgment to interpret the results. It is labor-intensive and subjective and not suited to high-volume testing.

Solid-phase immunoassays

Solid-phase immunoassays (SPIAs) have largely replaced other methods of serologic testing in routine clinical laboratories, and they are frequently performed on automated, high-throughput instruments. The term "solid-phase immunoassay" refers to the immobilization of an antigen or antibody on a solid phase such as a microtiter plate or microparticle. These assays take several forms (Fig. 5.3), but the most common type of SPIA is the non-competitive indirect enzyme immunoassay (EIA) (Fig. 5.3A). In indirect EIAs, patient serum is allowed to react with immobilized viral antigens, and the presence of bound virus-specific antibodies is detected using an MAb coupled to an enzyme (e.g., alkaline phosphatase or horseradish peroxidase) that, in the presence of an appropriate substrate, produces a colorimetric or fluorescent signal whose intensity is directly related to the amount of antibody present in the original sample. While the enzyme-coupled antibody could be specific for any isotype, nearly all non-competitive EIAs use an IgG-specific antibody for detection. Most commonly used assays to detect antivirus IgM have a different format (see below).

In competitive SPIAs, viral antigens are immobilized, but a known amount of enzyme-coupled virus-specific antibody is included in the reaction mixture (Fig. 5.3B). Virus-specific antibody present in the patient serum competes with the enzyme-coupled antibody, and the resulting signal is indirectly related to the amount of antibody present in the patient's specimen. This type of assay is able to detect both IgM and IgG in the same assay, and is most commonly used in the HBV core total

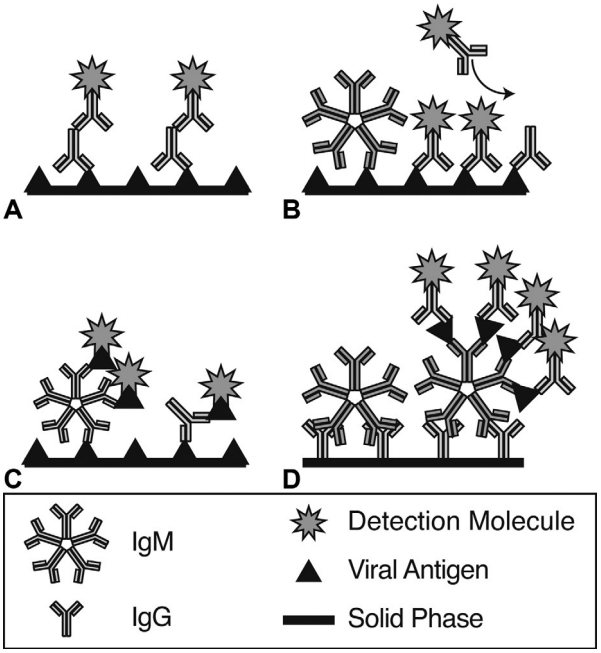

Fig. 5.3. Common immunoassay (IA) formats. **A**, Indirect IA; **B**, competitive IA; **C**, double-antigen sandwich IA; **D**, immunoglobulin (Ig) M class capture IA.

antibody and hepatitis A virus (HAV) total antibody assays. Another format that allows detection of IgM as well as IgG is the "double-antigen sandwich" (Fig. 5.3C). Immobilized viral antigens bind antiviral antibodies in the patient sample. The bound antibodies are then detected by an enzyme-coupled viral antigen rather than by an anti-IgG conjugate, followed by the addition of a substrate.

A number of advances in immunoassays over the past 20 years are evident in the evolution of human immunodeficiency virus (HIV) serologic tests. The first generation of HIV assays were performed manually using microplates or beads in tubes, and the target antigens were derived from whole-virus lysates. To increase specificity, second-generation assays used recombinant proteins or synthetic peptides as antigen sources, and included HIV-2. Third-generation HIV assays include antigens to detect group O variants of HIV-1, and these employ the double-antigen sandwich design that detects IgM as well as IgG and reduces the antibody-negative window period to 3–4 weeks (Burgisser et al., 1996). Finally, the fourth-generation HIV assays, which have been available in Europe but first became available in the United States in 2010, detect HIV p24 antigen as well as anti-HIV-1/HIV-2 antibodies (Miedouge et al., 2011). Antigen–antibody combination assays allow for early detection of infection before antibodies emerge and shorten the window period to approximately 2 weeks (Sickinger et al., 2004; Branson, 2007).

Another major advance has been the use of random-access instruments, commonly used in chemistry laboratories, for infectious disease serology. For traditional microplate-based serology assays, samples are batch-tested on a plate and progress as a unit through the reagent additions, incubations, washes, and detection steps in order to reduce costs and increase efficiency. One batch must be completed, which requires 3–4 hours, before the next batch can be loaded on to the instrument. Samples may be held in a queue for several days until a sufficiently large number of specimens are available to test. In contrast, random-access platforms allow for samples tested for a variety of analytes to be loaded and tested continuously, dramatically reducing turnaround time. Random access or "on-demand" methods often use chemiluminescent labels which produce light when excited by chemical energy. Chemiluminescent immunoassays (CLIA) are more sensitive, less prone to interference, and have a lower background signal than EIA or IF (Kim et al., 2008). Random-access instruments are also being developed for molecular testing (see below). EIAs are still performed manually for some analytes because kits for use on automated instruments are not yet available.

IgM antibody assays

False-positive and false-negative results are a major concern when testing for virus-specific IgM. High levels of IgG can block the binding of IgM to viral antigen on the solid phase, leading to false-negative results. Sera containing rheumatoid factor (an IgM antibody with anti-IgG specificity) are prone to false-positive results. Methods to remove interfering IgG and rheumatoid factor from sera prior to testing have been developed (Martins et al., 1995). An alternative to pretreatment of serum is the IgM class capture assay, also called reverse capture or μ capture (Fig. 5.3D). In this method, anti-IgM is immobilized on the solid phase, and binds all IgM antibodies in the patient's serum regardless of specificity. Viral antigens are then incubated with the bound IgM, and enzyme-coupled virus-specific antibodies are used to detect bound viral antigens. IgM capture assays are considered the most sensitive and specific format (Besselaar et al., 1989). However, for West Nile virus in particular, an additional background subtraction step is needed to reduce non-specific reactivity and avoid false-positive results (Rawlins et al., 2007).

Latex agglutination

Latex agglutination assays are among the simplest to perform and are widely used in the diagnosis of acute infectious mononucleosis. In latex agglutination, latex particles are coated with target antigens, and, when

incubated in the presence of patient serum containing specific antibodies, the latex particles form clumps that become visible to the naked eye. Latex agglutination tests using Paul–Bunnell antigen have largely supplanted the original monospot test for heterophile antibodies that used bovine or horse red blood cells following adsorption with guinea pig kidney (Bruu et al., 2000). Latex agglutination tests are subject to a "prozone effect" wherein high levels of antibody generate a falsely negative result due to saturation of all antibody-binding sites and minimal agglutination.

Lateral flow immunochromatography

Lateral flow immunochromatography (IC) assays have been developed for serologic testing, and some are Food and Drug Administration (FDA)-approved as point-of-care tests. A patient specimen is mixed with a test buffer, and this mixture is either applied to the sample loading pad or the test device is inserted into the sample buffer mixture, depending on the kit. Antibody present in the sample reacts with labeled antigen on the strip; antigen–antibody–detector conjugate complexes are drawn along the test strip by capillary action, and are captured by specific antibodies in the test area, giving rise to a visible line or spot. Rapid HIV antibody tests are IC tests that detect anti-HIV antibodies in serum, saliva, or oral mucosal transudate (Delaney et al., 2011). Recently, similar tests have been developed for hepatitis C virus (HCV) (Lee et al., 2011).

Immunoblot assays

In a Western blot, the viral proteins from an infected cell lysate are loaded on to a gel, subjected to electrophoresis, separated by molecular weight, and then transferred to a nitrocellulose membrane and immobilized. For the recombinant immunoblot assay (RIBA), also called strip immunoblot, recombinant or synthetic peptides are directly applied to the membrane. Patient serum is then incubated with the membrane, followed by incubation with anti-IgG coupled to an enzyme. Addition of substrate leads to deposition of colored substrate at the sites of antibody binding, and specific antibody–antigen reactivity can be visualized. Criteria have been established for the interpretation of immunoblots, and reports often include the identity and intensity of reactive bands. Western blots has been commonly used for confirming screening assays for HIV-1 and -2, human T-lymphotrophic virus (HTLV I/II), and for differentiating HSV-1 from HSV-2 infection (Wald and Ashley-Morrow, 2002; Mahajan et al., 2010). RIBA was used for confirmation of HCV screening tests until the manufacturer ceased production in 2013 (Damen et al., 1995).

Electron microscopy

Viruses are too small to be seen by conventional light microscopy, and electron microscopy (EM) is the only technique that allows for the direct visualization of viruses (Biel and Gelderblom, 1999). Recognition of viral morphology and ultrastructural details of virus-infected cells is the basis of viral identification by EM and requires substantial expertise. The two EM methods used for viral identification are negative staining and thin sectioning. Contrast between viruses and the background in EM images depends on differences in the electron density of the structures being examined. In negative staining, electron-dense, fine-grained, heavy-metal salts are added to samples and staining material accumulates around the virus and within features of the viral capsid or envelope. Areas lacking stain appear white against a black background, and viral structures that have accumulated stain may appear as different shades of gray. Approximately 10^6 virions/mL are needed for visualization by negative staining. Some specimens may require high-speed centrifugation to concentrate viruses, and vesicular fluid, stool, CSF, tissue culture supernatants, urine, and serum are all amenable to EM analysis. In immune EM, addition of specific antibody leads to aggregation of viral particles, facilitates visualization, and led to the discovery of many gastroenteritis viruses in stool extracts. Thin sectioning is performed on tissue biopsies or cells when histopathologic findings are suggestive of a viral infection. After fixation and embedding in polymer resin, samples are sectioned, analogous to paraffin-embedded tissues in pathology, but the prepared sections are much thinner. Staining is then performed using heavy-metal salts. EM for viral diagnosis is offered by very few laboratories. However, EM has proved invaluable in the identification of novel and unculturable viruses, including the recently described severe acute respiratory syndrome (SARS) coronavirus (Drosten et al., 2003; Ksiazek et al., 2003).

Histopathology and cytology

Virus particles cannot be seen by light microscopy but viral infections can be detected in tissue specimens using both morphologic features and special staining techniques. Viral replication can distort the normal cellular and nuclear architecture, giving rise to the appearance of "ground-glass" nuclei and multinucleate cells, or can form cytoplasmic and/or nuclear inclusions characteristic of a particular viral infection. More specific tests may be performed, such as immunohistochemistry and *in situ* hybridization (ISH), to identify viruses in tissue. Histopathologic diagnosis of viral infections is described in detail in Chapter 8.

Viral antigen detection

The detection of viral antigens in clinical samples provides direct evidence of viral infection. In contrast to culture and PCR, antigen detection methods do not amplify the target virus, making them inherently less sensitive. Another limitation is the antigenic variability of some targeted viruses, which can vary from year to year, especially for influenza; thus the performance of a particular kit may vary from year to year. When a novel variant emerges, such as pandemic influenza A H1N1, the performance of antigen tests for the detection of the new strain is not known, and indeed for pandemic H1N1 influenza, was lower than for seasonal influenza (Centers for Disease Control and Prevention, 2009; Ginocchio et al., 2009; Landry and Ferguson, 2010). In these cases, clinical diagnosis and other testing modalities are essential.

Many of the assay formats used to detect antibodies are also used to detect viral antigens.

SPIA

Automated SPIAs are widely used to detect HBV antigens (HBsAg and HBeAg), and the fourth generation of automated CLIA HIV antibody assays also include the detection of HIV p24 antigen. Rotavirus, which is shed in high titer in stool in infants, is commonly detected by manual microtiter–plate enzyme-linked immunosorbent assay (ELISA) (Dennehy et al., 1988) or by automated immunoassays (Dennehy et al., 1994).

Immunofluorescence

Direct immunofluorescent assays (DFA) for the detection of viral antigens in infected cells are widely used for the diagnosis of respiratory virus infections and herpetic skin lesions. IF detection of CMV matrix protein pp65 in the nuclei of peripheral blood leukocytes (PBL; CMV pp65 antigenemia) was the first CMV viral load test in blood and retains some advantages over molecular methods (van der Bij et al., 1988; Gerna et al., 1992; Landry and Ferguson, 1993). DFA can be performed on many cellular sample types, especially nasopharyngeal washes, aspirates or swabs, bronchoalveloar lavage, skin and mucosal lesions, PBL and tissue biopsies. Cells collected from a patient are pelleted by centrifugation, applied to glass slides, fixed, stained with antibodies coupled to fluorophores, then examined under a fluorescence microscope for virus-specific staining (Fig. 5.4 A and B). The color and intensity of the stain, the distribution of viral proteins within the infected cell, the infection of specific cell types, and the number of positive cells all impact the interpretation (Landry, 2009b). For CMV antigenemia, 200 000 PBL

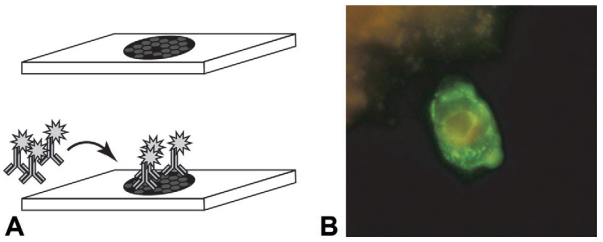

Fig. 5.4. Direct immunofluorescence (DFA) detects viral antigens in patient specimens. **A**, Cells from patient sample affixed to microscope slide (upper panel) followed by addition of fluorophore-labeled antiviral monoclonal antibody (lower panel). **B,** Herpes simplex virus-positive basal epithelial cell from a skin lesion showing apple-green fluorescence (400× magnification).

are applied per slide and the number of positive cells is counted. Some reagents are comprised of a pool of MAbs to different viruses that cause a similar clinical syndrome; thus a single reagent can screen for multiple viruses in a single cell spot. Examples include respiratory virus screening reagents for seven or eight respiratory viruses (RSV, influenza A and B, parainfluenza types 1, 2, 3, adenovirus, and hMPV) (Landry and Ferguson, 2000). If the screening reagent is positive, additional slides are stained to identify the specific pathogen, using either single fluorescein-labeled antibodies or dual-antibody reagents with two different fluorophore labels. Dual fluorophore-labeled antibody pools can also be used to detect and differentiate between HSV and VZV (Brumback et al., 1993).

Advantages of DFA over other antigen detection methods include greater sensitivity, ability to assess sample adequacy (i.e., sufficient numbers of target cells), and ability to detect multiple viruses in a single test (Chan et al., 2001). Limitations include the need for sufficient target cells, a fluorescence microscope, a dark room, meticulous technique in slide preparation, and expertise in interpretation to distinguish specific from non-specific staining (Landry and Ferguson, 2010). Assay time is 2 hours, which is longer than simpler rapid tests (Landry, 2009b). Nevertheless, DFA has been shown to improve patient care, provide cost savings, and reduce use of antibiotics (Barenfanger et al., 2000).

Lateral flow immunochromatography

IC assays are available for rotavirus (Dennehy et al., 1999) but are most widely used for the detection of influenza A and B and RSV. Some are approved as point-of-care tests. The sample is added to the sample pad or port, and viral antigen, if present, is bound by labeled antiviral antibodies. Antigen–antibody complexes move along the strip by capillary action until captured in the test area by virus-specific antibodies, leading to the appearance of a

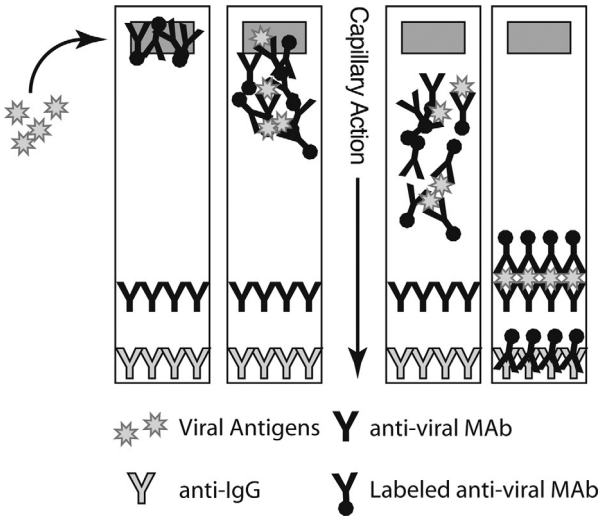

Fig. 5.5. Immunochromatography for the detection of viral antigens. The patient specimen is applied to a defined area that contains antiviral antibodies labeled with a detection molecule. Labeled antibodies with or without bound antigen are drawn along the test strip through capillary action. Antiviral monoclonal antibody (MAb) and anti-IgG are immobilized at the distal end of the test strip in well-demarcated areas. Viral antigens mediate the retention of labeled antiviral antibodies at the test strip, and anti-IgG binds residual labeled antibodies present. This leads to visible lines appearing at both the test and control locations when viral antigens are present (positive test) or the control location only when antigens are absent (negative test).

visible band (Fig. 5.5). Excess labeled antibodies continue to migrate up the strip until captured in the control area by anti-IgG. IC assays have largely replaced membrane EIA and other rapid test formats since IC simply requires the addition of the sample followed by setting of a timer to 10–20 minutes depending on the test. In contrast to EIA, there are no reagent additions or wash steps (Newton et al., 2002; Landry et al., 2004). IC tests require no equipment and can be performed by non-laboratory personnel. Limitations of IC include low sensitivity compared to other detection methods (Centers for Disease Control and Prevention, 2009; Ginocchio et al., 2009; Uyeki et al., 2009) and ability to detect only one or two different viruses (influenza A and B, or RSV). Improper collection devices, transport conditions, sample application technique, or failure to read results promptly all contribute to false-positive or negative IC results. Newer methods utilize a small instrument to enhance sensitivity and avoid errors in interpretation and result transcription.

Latex agglutination

Latex beads coated with virus-specific antibodies have been used for the detection of viral antigen, such as for rotavirus and enteric adenovirus in stools of infants, and more recently for norovirus (De Góes et al., 2008; Lee et al., 2010). When mixed with stool extracts, antibody-coated beads will clump if viral antigen is present.

Nucleic acid detection

Early molecular methods were more esoteric and expensive, but not more sensitive, than antigen and culture methods, and thus were not widely applied in the clinical arena. The first clinical viral molecular tests used non-amplified nucleic acid hybridization with radioisotope-labeled probes to detect human papillomavirus (HPV) DNA in cervical samples, as no other test for HPV was available. ISH using non-radioactive probes on tissue samples, such as for EBV, was the next advance. Hybrid capture, whereby nucleic acid hybrids in solution were captured by MAbs and detected, was subsequently introduced for HBV DNA quantification.

In contrast to the early non-amplified assays, PCR amplifies target nucleic acid by a million-fold, greatly increasing sensitivity, and has revolutionized viral diagnostics, especially for the diagnosis of CNS infections. A number of nucleic acid amplification test (NAAT) strategies are now available, but PCR remains the most widely used. Quantitation of viral nucleic acids, or determining viral load, is possible and is essential for the management of viral infections such as HIV, CMV, HBV, and HCV. The complex nature of molecular testing and lack of FDA-approved kits for many viruses has limited testing to large academic centers and reference laboratories, but recent innovations are expanding the availability of molecular testing (Table 5.3).

Conventional PCR

Conventional PCR consists of three steps: extraction and purification of nucleic acid, amplification of target sequences using specific primers and DNA polymerase, and detection of amplified fragments or "amplicons." Molecular assays rely upon the complementary base-pairing that is inherent to nucleic acids to effect this amplification. Templates for amplification can either be DNA or RNA (Saiki et al., 1985, 1988; Mullis and Faloona, 1987).

Extraction

Before an amplified test can be performed, the viral nucleic acids must be isolated and inhibitory substances removed. Nucleic acid extraction methods range from manual to fully automated, and can process individual samples or handle high-throughput processing of multiple samples at a time. Methods vary in ability to extract

Table 5.3

Common molecular methods

Technique	Target	Enzyme chemistry	Amplified product	Detection	Applications*	Commercial systems	Comments
PCR or RT-PCR	DNA or RNA	Taq DNA polymerase, plus reverse transcriptase for RNA viruses	DNA	Gel electrophoresis, hybridization, real-time methods (e.g. TaqMan probes, SYBR green), fluorescent microspheres, microarray, melting curves, electrochemical	HIV, HCV, HBV, CMV viral load tests, enterovirus, respiratory viruses, HSV 1, 2, published protocols available for most viruses	Roche COBAS, Abbott m2000, Cepheid Gene Xpert, Roche LightCycler, Cepheid Smartcycler, Applied Biosystems, Luminex xTAG, GenMark eSensor, Biofire FilmArray, Nanosphere Verigene, Focus Simplexa	Several integrated platforms available‡; most widely used for "home brew" assays; highly multiplexed assays can detect over 20 viruses in a single reaction
NASBA	RNA preferred	T7 RNA polymerase, reverse transcriptase, and RNase H	mRNA	Molecular beacons (real-time)	HIV, enterovirus	bioMerieux NucliSENS	Isothermal; less prone to carryover contamination
TMA	RNA preferred	T7 RNA polymerase, reverse transcriptase with RNase H activity	mRNA	Hybridization protection assay / dual kinetic assay	HCV, HIV qualitative HIV, HCV, HBV, WNV†	Gen-Probe Procleix, Roche	Isothermal; less prone to carryover contamination
bDNA	RNA or DNA	Series of target, secondary branched, and tertiary enzyme-labeled probes	Probe signal	Fluorescence	HCV, HIV viral load	Siemens Versant	Simpler to perform than PCR; less prone to carryover contamination

*Food and Drug Administration (FDA)-approved assays for *in vitro* diagnostics.
†FDA-approved assays for donor screening.
‡"Integrated" refers to integration of all reaction steps, allowing the operator simply to add the sample and walk away.
bDNA, branched DNA; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; TMA, transcription-mediated amplification; WNV, West Nile virus.

RNA, DNA, or total nucleic acid, may perform differently for different sample types, and vary in degree of concentration of nucleic acid of the final extract relative to input sample. Thus the extraction method used can greatly impact the sensitivity of the final result.

Traditional nucleic acid isolation techniques using phenol/chloroform have been supplanted by other methods. Single-sample spin column kits isolate either DNA or RNA by binding to a resin following alkaline lysis of cells. After washing, nucleic acids are eluted into an aqueous solution. Binding of nucleic acids to silica beads in solution, followed by washes and centrifugation to remove impurities, then elution into a buffer is another common strategy. These kits require significant hands-on time and are not amenable to high-throughput testing.

A number of automated extraction devices are now available that use magnetic silica for nucleic acid isolation and can be used in conjunction with many different NAAT. Some instruments have extraction kits for specific specimen types, including sputum, tissue, body fluids, and swabs. Other platforms use a single protocol for all sample types and can extract both DNA and RNA (total nucleic acid) (Yang et al., 2011).

Amplification

PCR uses small DNA primers (approximately 20 base-pairs (bp)) that bind to complementary regions of the target nucleic acid sequence. The temperature at which the DNA primers anneal to the target DNA sequence depends on the length of the primers and the sequence targeted, and in most cases is around 55–60 °C. PCR uses a thermostable DNA polymerase, originally isolated from *Thermus aquaticus* (Taq), a bacterium that lives in hot springs, whose optimal activity is around 72 °C. However, Taq polymerase is relatively stable at the higher temperature (approximately 95 °C) required to "melt" the larger strands of double-stranded DNA (dsDNA) generated by PCR into single strands. The thermostability of Taq polymerase allows for multiple amplification cycles consisting of raising the temperature to 95 °C to melt dsDNA, lowering the temperature to 55 °C to allow primer annealing, and raising the temperature to 72 °C for polymerase activity. When this is repeated for 30–45 cycles, target DNA doubles with each cycle, and exponential expansion of the original nucleic acid sequence occurs, facilitating its detection.

Since DNA is the template for PCR amplification, reverse transcriptase (RT) must be used to synthesize a complementary DNA (cDNA) segment from an RNA template, such as for RNA viruses, prior to PCR. When PCR is performed on cDNA generated by RT, it is referred to as RT-PCR; this should not be confused with real-time PCR (see below).

PCR assays for the same virus vary tremendously in the gene target, the primer sequences selected, the reaction components, and amplification conditions. Some assays intend to be broadly reactive, and others to be selective for certain virus genotypes or serotypes.

Detection

As originally described for conventional or endpoint PCR, amplified products are detected by agarose gel electrophoresis after amplification is complete. A small volume of the PCR reaction is loaded on to an agarose gel impregnated with ethidium bromide, a highly carcinogenic fluorescent molecule that tightly binds DNA, and products are separated based on the length of the dsDNA fragment. Amplified PCR products are then visualized as distinct bands of an appropriate size after illumination with ultraviolet light. Alternatively, amplicons can be transferred by Southern blot or spotted directly on to nitrocellulose for hybridization with a specific probe. Conventional PCR requires relatively inexpensive thermocyclers and gel visualization systems. However, vials must be opened in order to remove an aliquot for detection, and this can aerosolize DNA amplicons, leading to contamination of instruments, surfaces, gloves, and clothing. This presents a high risk for contamination of subsequent PCR reactions, and unidirectional work flow protocols using three separate rooms for extraction, amplification, and detection are widely used in molecular laboratories. Another common strategy is substitution of uracil (dUTP) for thymidine (dTTP) in the reaction master mix to generate DNA amplicons containing uracil instead of thymidine. Subsequent addition of uracil-*N*-glycosylase (UNG) to all reactions prior to PCR will prevent uracil-containing amplicons, but not native DNA, from serving as templates for amplification (Pang et al., 1992).

Quantitation of conventional PCR requires PCR of multiple dilutions of starting material or serial dilutions of amplicons followed by plate hybridization. Quantitative viral load testing for HIV, HBV, and HCV was originally performed in this manner, but has been supplanted by alternative techniques in most cases.

Real-time PCR

Real-time PCR methods have had a major impact on diagnostic testing. Real-time PCR combines amplification and detection in one step, which shortens assay time, reduces amplicon cross-contamination because the reaction tube is not opened, and allows visualization of amplification results as they are unfolding in "real time." Real-time PCR methods commonly use fluorescent

DNA-binding dyes such as SYBR green, analogous to ethidium bromide in gel electrophoresis, or hybridization with fluorescent DNA probes (Livak et al., 1995; Wittwer et al., 1997). Thus, real-time assays require the use of thermocyclers with built-in light sources, filters, and detectors.

SYBR green and similar dyes become fluorescent upon DNA binding and have a higher affinity for dsDNA molecules. As the amount of dsDNA created by PCR increases, the fluorescent signal arising from the dyes also increases. These assays do not require a complementary DNA probe for detection, and could be beneficial for the detection of viruses with sequence variability. However, the dyes bind all dsDNA, including primer dimers and non-specifically amplified material, and this also renders SYBR dyes less amenable to multiplex assay development (see below). Specificity is determined by determining the melting temperature (T_m) of the DNA amplicons, which is unique to the nucleic acid sequence (Espy et al., 2006).

The most common real-time method using DNA probes relies upon the 5'-3' exonuclease activity of Taq polymerase and these are commonly referred to as TaqMan assays. TaqMan assays use a DNA probe that is complementary to a region of DNA in the amplified PCR product and labeled at the 5' and 3' ends with fluorophores of different excitation and emission spectra (a reporter and quencher). When the probe is intact, fluorescence is quenched. This is referred to as fluorescence resonance energy transfer (FRET). When the target sequence is present, primers and probe anneal, and, as the primers extend, degradation of the TaqMan probe (also called hydrolysis probe) leads to separation of the reporter fluorophore from the quencher (Fig. 5.6A). With each ensuing cycle of amplification, fluorescence accumulates until it crosses the threshold to positive at a specific cycle of amplification, also known as the cycle threshold or Ct value (Fig. 5.6B). The Ct value provides an indication of the amount of viral target in the original sample: the lower the Ct value, the higher the viral load. When standards of known viral concentration are run in parallel with patient samples, absolute values for viral load can be calculated using a standard curve (Figs 5.6C and 5.7). FRET hybridzation probes, also referred to as LightCycler probes, and molecular beacons are two other common probe detection formats used in real-time PCR (Espy et al., 2006).

For qualitative assays with results reported simply as "positive" or "negative," the Ct value can provide important additional information for clinical management, such as following response to treatment, if serial samples are tested, or determining which of two viruses detected is the predominant pathogen. Communication with the laboratory is encouraged for proper interpretation of results.

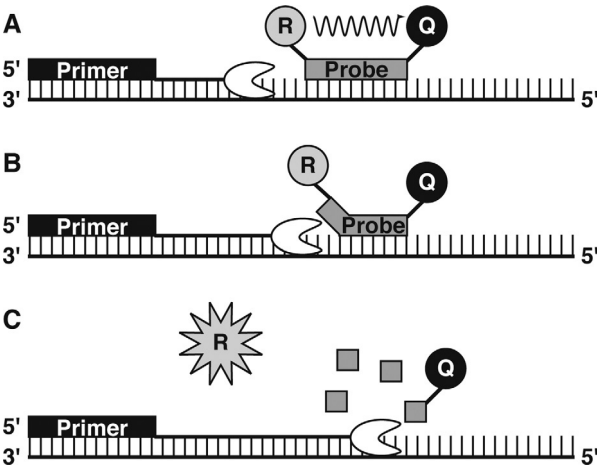

Fig. 5.6. Real-time polymerase chain reaction (PCR) using TaqMan probes. Specific primers bind to complementary basepairs on the DNA template. A probe labeled with a fluorescent reporter molecule (R) and a quencher (Q) binds to a complementary region between the forward and reverse PCR primers. While the probe is intact, fluorescent emissions by the reporter molecule are quenched. During the PCR reaction and Taq polymerase-mediated DNA elongation, the 5' exonuclease activity of Taq polymerase degrades the intact probe. Separation of the reporter from the quencher allows reporter molecule fluorescence to be detected. With each cycle of amplification and probe degradation, fluorescent signal increases.

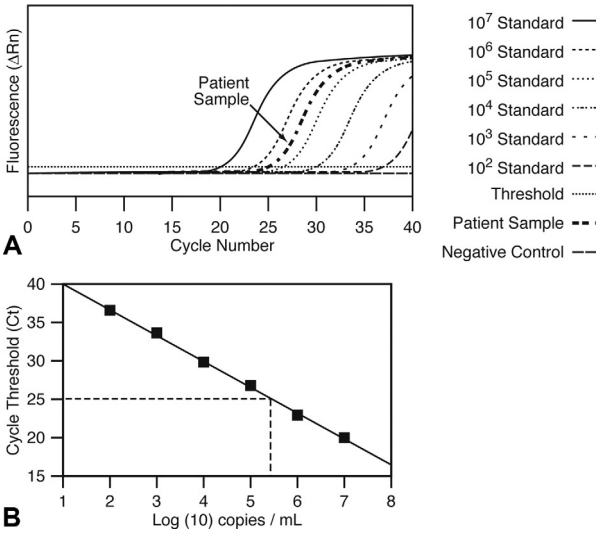

Fig. 5.7. Real-time polymerase chain reaction (PCR) quantitation of viral load. **A,** Unknown patient specimen (arrow) and standards of known concentration are assayed. **B,** A plot of known standard concentrations versus cycle threshold (Ct) value allows for the determination of viral load in the patient specimen. In this example, the patient Ct value of 25 corresponds to a viral load of 250 000 copies per mL.

Both SYBR green and TaqMan assays can be performed on a variety of real-time PCR instruments, and these may vary in the speed at which they cycle, the reaction vessel used, and the number of samples that can be tested in a single run. The LightCycler instrument utilizes glass capillaries as reaction vessels to reduce the time required to raise and lower temperature for each amplification cycle, and thus shorten assay time. The Smartcycler also has a unique reaction vessel that allows faster cycling; in addition, each reaction in the 16-sample unit works as an independent thermocycler, so samples do not need to be batch-tested. Applied Biosystems instruments such as the ABI 7500 use 96-well plates for higher throughput, and a rapid-cycling option (ABI 7500 Fast Dx) that utilizes smaller reaction volumes is available. A current focus in test development is greater automation, for both high-volume and low-volume testing laboratories.

Multiplex methods

Multiplex tests detect the presence of multiple viruses in a single reaction. While greatly increasing efficiency of testing, multiplex assays can be less sensitive than single tests. Real-time PCR instruments incorporate multiple light sources and filters that allow for the simultaneous monitoring of three to five different fluorophores. This has allowed the development of multiplexed real-time assays, some of which are commercially available, such as several kits that detect influenza A and B and RSV as well as an internal control in a single test (Legoff et al., 2008; Liao et al., 2009).

To multiplex more than four targets, conventional PCR is used and the number of viral targets is limited by the resolution of the detection method. An early commercial test for six herpesviruses in CSF by Argene used conventional PCR for HSV-1, HSV-2, VZV, EBV, CMV and human herpesvirus-6 (HHV-6) followed by plate hybridization, but was available in the United States as a research use only (RUO) test (Calvario et al., 2002). The FDA-approved xTAG respiratory virus panel is the prototypical highly multiplexed assay, and it uses conventional PCR and virus-specific oligonucleotide TAGs that bind to fluorescent microspheres. The "color" of the microsphere corresponds to an individual virus, and the labeled DNA strand indicates whether an amplified product is present. Detection uses a Luminex instrument in which microfluidics and laser systems analogous to those used in flow cytometry monitor individual microspheres for the presence of amplified nucleic acid. The system is multiplexed for the detection of 12–20 respiratory virus targets in one reaction (Mahony et al., 2007; Pabbaraju et al., 2008).

Highly multiplexed assays from Idaho Technologies (now BioFire) and GenMark have recently been FDA-approved for the detection of respiratory pathogens in the United States. These three highly multiplexed testing platforms differ dramatically in their extraction, amplification, and/or detection. The BioFire FilmArray system uses a self-contained pouch that combines extraction, two sequential PCR reactions (nested PCR), and detection in a single unit. Results take approximately 1 hour, but throughput is limited to one sample per instrument per hour. Like the Luminex xTag system, the GenMark respiratory virus panel requires separate extraction and endpoint PCR amplification, but this system uses their proprietary eSensor electrochemical detection method. Comparative studies are limited, but the GenMark assay demonstrates sensitivity comparable to optimized individual PCR assays, while the FilmArray was somewhat less sensitive (Loeffelholz et al., 2011; Babady et al., 2012; Pierce and Hodinka, 2012; Pierce et al., 2012). The FilmArray system is able to be performed by staff with a wide array of skill levels and generates a final report in approximately 1 hour, in contrast to 8–16 hours for the Luminex and GenMark assays (Xu et al., 2013).

Nanosphere has developed a "bead microarray" technology capable of high-order multiplexing, but the available respiratory virus panel is limited to seven analytes (Alby et al., 2013). Other highly multiplexed commercial tests for respiratory viruses use a variety of strategies for the detection and differentiation of amplified products, such as microarrays with an automated analyzer, automated endpoint melting curves, capillary electrophoresis, and other novel strategies (Marshall et al., 2007; Nolte et al., 2007; Raymond et al., 2009). These highly multiplexed methods have almost exclusively been developed for the detection of respiratory pathogens, but the underlying technologies can be applied to other body sites and specimens in which clinical syndromes could be caused by a variety of difficult-to-identify and/or distinguish pathogens such as gastrointestinal infections.

Non-PCR molecular techniques

Several commercial platforms use non-PCR molecular techniques for viral diagnosis (Table 5.3). Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are highly related and demonstrate comparable performance to PCR assays (Guatelli et al., 1990; Giachetti et al., 2002). NASBA and TMA both amplify an RNA target sequence, but, in their commercial applications, use different techniques to detect the amplified material, molecular beacons and hybridization protection assays (HPA), respectively (Giachetti et al., 2002; Landry et al., 2003). Like TaqMan probes, molecular beacons

are oligonucleotide probes with reporter and quencher fluorophores at the 3' and 5' ends. Molecular beacons have areas of complementary DNA within the probe that lead to hairpin formation placing the fluorophores in close proximity leading to FRET and no signal. Upon DNA binding, the molecular probe unfolds, and the FRET is eliminated, leading to increasing signal. NASBA with molecular beacons is a real-time technique (Leone et al., 1998). HPA uses a nucleic acid-binding acridinium ester that, in its unbound form, is inactivated by chemical treatment. When material is amplified by TMA, the acridinium ester hybridizes to the double-stranded nucleic acid and is protected from inactivation. Addition of an activating compound leads to the production of visible light from protected, nucleic acid-associated acridinium ester that is read by the instrument. HPA is an endpoint detection technique rather than real-time.

Branched DNA (bDNA) assays do not amplify the nucleic acids present in the original sample, but these molecules serve as a template upon which multiple layers of complementary probes are built (Dewar et al., 1994; Mellors et al., 1996). The final layer of oligonucleotides is coupled to an enzyme that, in the presence of an appropriate substrate, generates a fluorescent signal. bDNA is not susceptible to amplicon carryover, is less technically demanding, and is simpler to implement than current PCR assays.

These techniques do not lend themselves to the development of "homebrew" (see below) assays without substantial support. Additionally, other molecular methodologies have been described, and some of these are used in other areas of clinical diagnostics. However, none has been developed for viral diagnosis.

Newer molecular platforms

Increasingly, manufacturers are developing simpler, walk-away testing platforms to broaden the availability of molecular testing to laboratories performing lower-complexity testing. Some systems are entirely self-contained and closed to the development of laboratory developed assays, while others are more open to outside assays but require a greater degree of technical oversight. It is theoretically possible that existing assays could be adapted to run on these platforms, but it is likely that manufacturer-produced assays will be the primary focus of development.

The GeneXpert system (Cepheid) was originally implemented for anthrax screening in post offices and uses cartridges to which a sample is added that contains all of the reagents and fluidics needed for nucleic acid extraction and real-time PCR. It was the first system to fully automate and integrate all steps from sample preparation to final report. It is random-access, with results available in 1 hour. An assay for enterovirus in CSF is FDA-approved on this system (Kost et al., 2007; Sefers et al., 2009). Initial instruments could test one, four, or 16 samples in a "walk-away" format, but a new instrument can test 48 or more depending on instrument configuration.

While the GeneXpert and FilmArray systems use complex fluidics to incorporate nucleic acid extraction into a simple-to-use cartridge or pouch, respectively, Focus Diagnostics has developed a direct "sample-to-answer" assay for influenza A, influenza B, and RSV that relies upon a proprietary PCR reaction mix to minimize inhibitory effects of patient specimens, allowing detection in approximately 1 hour (Alby et al., 2013). A large number of analyte-specific reagents (ASR; see below) are also available for this platform, including assays for the detection of HSV-1, HSV-2, VZV, and CMV that can be run with or without extraction. Other fully automated systems using novel strategies have been developed. Some are now FDA-cleared and available from other manufacturers.

Viral diagnostic assays based on microarrays, mass spectrometric analysis of amplified nucleic acids, and next-generation sequencing are on the horizon (Wang et al., 2002; Ecker et al., 2008). It remains to be determined whether these techniques will be cost-effective and offer enough clinical benefit in terms of diagnostic utility, improvement in laboratory work flow, or patient management to become widely adopted. Microarrays and mass spectrometric assays may allow for the multiplexed detection of a greater variety of viruses without a loss in sensitivity, but these methods are complex and will require substantial commercial development. Sequencing is currently used for viral genotyping and assessment of antiviral resistance, and next-generation sequencing techniques are both quantitative and able to detect small subpopulations of resistant viruses that may be clinically relevant (Simen et al., 2009).

Genotype, phenotype, and antiviral resistance

To determine resistance or susceptibility to antiviral drugs, phenotypic or genotypic assays can be used. Phenotypic assays require growth of virus in the presence of increasing concentrations of drug, then determining the drug concentration that reduces infectivity by 50% (IC50). While HSV grows readily in culture, and phenotypic testing results can be obtained within several weeks, the slow growth of CMV can delay phenotypic resistance results for 2 months or more and has largely been replaced by genotypic testing (Chou et al., 1995; Tebas et al., 1995; Safrin et al., 1996).

Current HIV phenotypic testing utilizes recombinant virus assays in which the HIV-1 RT and protease genes

are amplified from the patient's plasma, ligated into a viral vector, then transfected into a suitable cell line. Changes in viral replication in the presence of drug are monitored. Phenotypic testing is essential for identifying new mutations associated with drug resistance, but for HIV is only available in specialized reference laboratories. Phenotypic results are often expressed as "fold resistance" compared to wild-type virus (Petropoulos et al., 2000).

Drug resistance genotyping involves PCR amplification of the genes targeted by antiviral agents, followed by sequencing to identify known drug resistance mutations. It is most commonly undertaken for HIV and CMV, but testing of HBV and influenza is becoming important as well (Olivero et al., 2006; Deyde et al., 2009). Genotypic assays can determine if known resistance mutations are present, but the significance of identified novel mutations is impossible to interpret (Bennett et al., 2009).

HCV genotyping merely identifies the patient's virus as genotype 1–6 in order to predict treatment response and determine length of therapy. It does not identify drug resistance mutations. In the most common HCV genotyping assay, the 5' non-coding and core regions of the viral genome are amplified and allowed to bind to immobilized oligonucleotide probes on test strips (Line Probe Assay). The pattern of DNA binding correlates with a particular virus genotype, and individual genes are not sequenced (Verbeeck et al., 2008).

Lack of standardization of quantitative molecular tests

The quantitation of viral nucleic acids by molecular assays is important for the management of several acute and chronic infections. Quantitative assays are frequently performed for HIV, HCV, CMV, EBV, BK virus, and adenovirus. Because of the importance of HIV and HCV viral loads in the management of affected patients, the World Health Organization has developed international standards against which all commercially available assays are calibrated (Saldanha et al., 2005; Glaubitz et al., 2011). Thus, a viral load obtained using one assay should be the same as a viral load obtained using a different platform, in any laboratory in the world.

However, no such standards exist for most other viruses, and the widespread use of homebrew assays makes comparisons between laboratories nearly impossible. Recent international studies of CMV and EBV viral load testing by transplant centers found differences in quantitation of 2–3 log10 copies/mL between laboratories testing the same samples (Pang et al., 2009; Preiksaitis et al., 2009). All aspects of the assay procedure (e.g., sample type, extraction method, molecular target, primer and probe sequences, master mix, amplification protocol) contribute to the quantitation of viral nucleic acid, and modification of any of these parameters could give a different result. An international standard for CMV has recently become available to both assay manufacturers and independent laboratories (Freyer et al., 2010). Efforts are under way to develop international standards for other viruses, but, until that time, quantitative results should be obtained from a single laboratory (Madej et al., 2010). If multiple laboratories must be used, careful correlation of viral load testing between the laboratories should be performed.

Regulatory requirements and availability of molecular testing

Regulations and test approval processes in different parts of the world affect test availability. In the United States, the FDA approves test kits. For *in vitro* diagnostic (IVD) use, kits must undergo rigorous testing by the companies that have developed them. Kit quality control is performed by the manufacturer. Implementation of IVD tests requires limited internal validation studies by the diagnostic laboratory.

In contrast to IVD tests, some assays may be commercially available only as ASR or RUO kits. Both contain the reagents required for performing the assay, but they have not undergone the testing required for FDA approval. Instead, the onus and expense for clinical validation studies are incurred by the diagnostic laboratory.

The final test category is "homebrew" or "laboratory-developed" tests. Homebrew tests are frequently based upon assays published in the literature. The performing laboratory is responsible for obtaining all the necessary reagents and must perform the quality control on these reagents. Additionally, homebrew tests must undergo an extensive internal validation process.

Many laboratories do not have the staffing or expertise to perform all of the steps required to implement homebrew tests, and assays using ASR or RUO kits may also be problematic given the validation requirements. However, despite the increasing number of commercial assays available for the detection of respiratory viruses, the number of viruses for which IVD molecular diagnostic kits are available is small. Thus, laboratories may have to rely upon less rapid tests or send samples to reference laboratories for more comprehensive testing. Internationally, different regulatory environments apply.

SAMPLE COLLECTION AND TRANSPORT

The samples to collect will be determined by the clinical syndrome, viruses in the differential, knowledge of their pathogenesis, the tests ordered, and the requirements of the testing laboratory (Tables 5.4 and 5.5). Laboratories provide collection guidelines appropriate to the tests that

Table 5.4

DNA viruses, syndromes, samples, tests

DNA viruses				
Viral family	Virus	Clinical syndrome	Sample to collect	Diagnostic technique
Herpesviridae	HSV-1, -2	Mucocutaneous lesions	Lesion swab	Culture, DFA, or NAAT
		Neonatal herpes, hepatitis, disseminated disease	Plasma, throat, CSF, lesion swabs	NAAT, culture; DFA
		Encephalitis, meningitis	CSF	NAAT
	VZV	Varicella, zoster	Lesion swab	DFA or NAAT
		Encephalitis, meningitis	CSF	NAAT
	EBV	Infectious mononucleosis	Serum	Serology, NAAT
		Lymphoma	Tissue, blood, CSF	Histopathology, NAAT
		Encephalitis, meningitis	Serum, CSF	Serology, NAAT
	CMV	Congenital	Urine, CSF	Culture, NAAT, serology
		Infectious mononucleosis	Blood	NAAT, serology
		Pneumonia, retinitis, esophagitis, colitis in compromised hosts	BAL, blood, tissue biopsy	NAAT, culture
		Encephalitis	CSF	NAAT
	HHV-6	Roseola	Serum	Serology
		Mononucleosis	Blood	NAAT, serology
		Encephalitis	CSF	NAAT
Polyomaviridae	JC virus	Progressive multifocal leukoencephalopathy	CSF, tissue	NAAT, histopathology, EM
	BK virus	Nephropathy	Plasma, urine, kidney biopsy	NAAT, histopathology, EM
		Hemorrhagic cystitis	Urine	NAAT
		Encephalitis	CSF	NAAT
Adenoviridae	Adenovirus	Respiratory, ocular, GI infections	NP or throat swab, BAL, ocular swab, stool	NAAT, DFA, culture
		Disseminated infection	Blood	NAAT
Parvoviridae	Parvovirus	Fifth disease, bone marrow suppression	Serum, bone marrow	Serology, NAAT, histopathology
Poxviridae	Variola	Vesiculopustular rash	Lesion swab or biopsy, tissue, blood	NAAT, EM, histopathology, culture, serology
	Vaccinia, monkeypox, cowpox	Vesiculopustular rash	Lesion swab or biopsy, tissue, blood	NAAT, EM, histopathology, culture, serology
Hepadnaviridae	HBV	Hepatitis, acute liver failure	Serum	Serology, antigen, NAAT

BAL, bronchoalveolar lavage; CMV, cytomegalovirus; CSF, cerebrospinal fluid; DFA, direct fluorescent assay; EBV, Epstein–Barr virus; EM, electron microscopy; GI, gastrointestinal; HBV, hepatitis B virus; HHV-6, human herpesvirus-6; HSV, herpes simplex virus; NAAT, nucleic acid amplification technique; NP, nasopharyngeal; VZV, varicella-zoster virus.

Table 5.5

RNA viruses, syndromes, samples, tests

RNA viruses				
Viral family	Virus	Clinical syndrome	Sample to collect	Diagnostic technique
Arenaviridae	LCMV*	Meningitis, encephalitis	CSF, serum	Serology, NAAT, culture
	Other Arenaviridae*†	Hemorrhagic fevers	Serum, tissue	Serology, culture
Bunyaviridae	California encephalitis virus (La Crosse), Jamestown Canyon virus*	Encephalitis, meningitis	CSF, serum	Serology, NAAT
	Rift Valley fever virus	Hemorrhagic fever, encephalitis	Blood, CSF	Serology, NAAT, isolation
Flaviviridae	WNV, JE, SLE, TBE, Powasson*	Encephalitis, meningitis	CSF, serum	Serology, NAAT
	Dengue fever virus*	Polyarthritis, rash, hemorrhagic fever	Serum	Serology, NAAT, culture
	Hepatitis C virus	Hepatitis, cryoglobulinemia, porphyria	Serum	Serology, NAAT
Orthomyxoviridae	Influenza	Respiratory infection	NP swab or aspirate, endotracheal aspirate, BAL	Antigen, NAAT, culture
Paramyxoviridae	Parainfluenza, respiratory syncytial, human metapneumovirus	Respiratory infection	NP swab or aspirate, endotracheal aspirate, BAL	Antigen, culture, NAAT
	Mumps	Parotitis, meningitis, deafness	Serum, saliva, CSF, urine	Serology, culture, NAAT
	Measles virus	Measles	Serum, NP swab, throat swab, urine	Serology, NAAT, culture
		Acute and postinfectious encephalitis	CSF	NAAT, Serology
		Subacute sclerosing panencephalitis	Brain tissue	Histopathology, EM
	Nipah*	Encephalitis	CSF	Serology, NAAT
	Hendra*	Pneumonia, encephalitis	BAL, CSF	Serology, NAAT
Picornaviridae	Enteroviruses	Meningitis, encephalitis, flaccid paralysis; a variety of other clinical diseases	CSF, stool	NAAT, culture
	Parechoviruses	Respiratory, neonatal sepsis, encephalitis, meningitis		NAAT, Culture
	Rhinoviruses	Respiratory infections	NP swab, BAL	NAAT

Retroviridae	HIV	Mononucleosis, acute retroviral syndrome, AIDS	Serum, plasma	Serology, NAAT
	HTLV*	Tropical spastic paraparesis, myelopathy, leukemia/lymphoma	Serum, CSF, PBMC	Serology, NAAT
Rhabdoviridae	Rabies	Rabies	Biopsy hair follicles nape of neck, CSF, serum, saliva, brain tissue	Antigen detection, NAAT, culture, serology; histopathology
Togaviridae	Rubella	Congenital	Serum, throat, urine	Serology, NAAT, culture
		Malaise, rash, arthralgias	Serum	Serology, NAAT
	WEE, EEE, VEE*	Encephalitis, meningitis	CSF, serum	Serology, NAAT
	Chikungunya virus*	Polyarthritis, rash	Serum	Serology, NAAT
Caliciviridae	Norovirus	Gastroenteritis, dehydration	Stool	NAAT, antigen, EM
Reoviridae	Rotavirus	Gastroenteritis, dehydration	Stool	Antigen, NAAT, EM

*Testing available at specialized reference laboratories.

†Other Arenaviruses include: Lassa fever virus, Argentine, Bolivian, and Venezulean hemorrhagic fever viruses, Sabia virus, Whitewater Arroyo virus.

AIDS, acquired immunodeficiency syndrome; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; DFA, direct fluorescent assay; EEE, Eastern equine encephalitis; EM, electron microscopy; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; JE, Japanese encephalitis; LCMV, lymphocytic choriomeningitis virus; NAAT, nucleic acid amplification test; NP, nasopharyngeal; PBMC, peripheral blood mononuclear cell; SLE, St. Louis encephalitis; TBE, tick-borne encephalitis; VEE, Venezuelan equine encephalitis; WEE, Western equine encephalitis; WNV, West Nile virus.

they offer, including sample type and volume, proper container, transport media or stabilizers if needed, transport temperature, and other special instructions. For optimal results, sample collection instructions should be strictly followed. Some general principles will be provided here.

Nasopharyngeal swabs should be inserted deep into the nasopharynx, past the point of resistance, and rotated to collect ciliated cells. Lesions should be unroofed with a sterile needle or scalpel and cells at the base of the lesion collected. Vesicle fluid, which contains high titers of virus, can also be absorbed on to the swab. For viruses shed in stool, stool samples, which contain more virus, are preferred to rectal swabs. Samples submitted for culture require the most careful handling in order to preserve viral infectivity. Swabs and tissue biopsies are placed in viral transport media with antimicrobials to prevent drying and reduce overgrowth of bacterial and fungal pathogens. Body fluids, aspirates, washes, and stool samples should be placed in sterile containers. Transport of specimens at 4 °C is preferred. Freezing samples at –20 °C leads to loss of viral infectivity and should be avoided. Freezing at –70 °C and subsequent thawing reduce infectivity for many enveloped viruses, and are only recommended if transport to the laboratory is delayed for several days. If rapid viral antigen detection kits are used, such as rapid influenza tests, the manufacturer's guidelines for sample type, collection device, and transport should be followed. Especially for non-amplified tests such as rapid flu tests, sample dilution in transport media should be minimized as dilution can further reduce already limited test sensitivity.

For molecular tests, viral nucleic acid, particularly DNA, is more stable than viral infectivity. However, quantitative molecular methods performed on blood require separation of serum or plasma from cells within 6 hours for accurate results. Likewise blood submitted for quantitative CMV pp65 antigenemia should be processed and cells fixed within 6 hours of collection for accurate results. For qualitative PCR tests, if the viral load is low, as occurs with most CSF samples, improper storage or delays in processing can give falsely negative results.

Serum samples for antibody studies can be transported at room temperature, but 4 °C is preferred. In contrast to samples for culture or PCR, freezing serum at –20 °C is acceptable for antibody studies. Paired samples should include the earliest available sample at onset of illness and no later than 5–7 days after onset, and a second sample collected 10 days to 4 weeks later to document seroconversion of IgG. Samples for IgM should be collected in the first week of illness; if negative, a second sample should be collected in the second week if suspicion remains high. When a rapid HIV test is performed at the point of care, whole blood from a fingerstick or oral mucosal transudate can be tested. If the sample is sent to the laboratory, blood collected in an anticoagulant, such as a lavender top tube, provides a more rapid result. If serum is submitted, such as a red top tube, instead of plasma, a delay of 30–60 minutes is required for the blood to clot before centrifugation and testing can be performed.

Although testing CSF is often considered the most direct means of diagnosis of CNS disease, some highly neuropathic viruses may be present in insufficient titer in CSF despite CNS tissue disease, and thus testing other samples can be essential. Examples include testing stool for enterovirus type 71 or poliovirus (Landry et al., 1995). Furthermore, some neurologic syndromes are immune-mediated, and virus may not be present in CSF but can be detected in peripheral sites, such as Guillain–Barré syndrome associated with many different viruses.

SUMMARY AND INTERPRETATION OF TEST RESULTS

Viral diagnostics has become an integral part of clinical management, with most results available within hours or a few days, rather than in weeks, as in the past. Each laboratory must select from an extensive and growing armamentarium of tests which tests to offer on site, based on the patient populations it serves, the need for rapid results, and the facilities, equipment, and expertise available.

The prominent role of conventional culture, long the gold standard, is declining. Rapid cultures have a more limited menu, but provide results in a clinically useful timeframe. Rapid antigen tests have allowed testing for influenza and RSV at hospitals and clinics without virology laboratories, and results can be available within minutes at the point of care. Although sensitivity of rapid tests is suboptimal, especially for viruses with antigen variability, in young children who shed high titers of virus their impact on reducing unnecessary antibiotics and other testing has been documented (Bonner et al., 2003).

DFA staining of clinical samples has enjoyed wider application in the past 20 years, from herpetic skin lesions, to respiratory viruses and CMV viral load in blood, due to high-quality commercial MAbs, pooled antibody reagents for multiplex screening of specimens, and the use of multiple fluorophore labels to differentiate antibodies. DFA is a very valuable method when done well, due to its relative speed, multiplex capability, and greater sensitivity compared to other rapid antigen tests. Indeed, a single positive cell can be detected. DFA has also been shown to improve care and reduce costs (Barenfanger et al., 2000). However, DFA requires a fluorescence microscope, extensive experience and judgment to differentiate specific from non-specific staining, attention to detail, and continual monitoring compared to culture or PCR to maintain quality. It is also manual, and all of these factors limit its availability.

Serology is still the test of choice for acute infections with arboviruses, lymphocytic choriomeningitis virus, measles, and rubella. When disease symptoms are immune-mediated, diagnosis by antibody response plays a central role. Examples include EBV, parvovirus B19, HAV, and HBV. Serologic tests have transitioned from manual, laboratory-developed tests using serial dilutions of serum, to commercial kits that test a single serum aliquot. Rather than whole virus grown in cell culture as the antigen source, current tests commonly use synthetic peptides or recombinant proteins to improve test specificity and reproducibility. Instrumentation allows automation, random access (on demand) or batched testing, and can interface directly with the laboratory computer system. In order to detect early HIV infections before IgG has developed, current SPIA formats allow detection of IgM as well as IgG in the same assay, or include antigen detection. Class capture has become the preferred test for IgM to reduce false positives and false negatives. Simple point-of-care tests for HIV antibody now allow results to be reported while the patient is still in the clinic. Nevertheless, it is important to remember that initial antibody tests may be negative, and a sample in the second week of illness, such as with West Nile virus IgM in CSF, may be needed to establish the diagnosis (Busch et al., 2008). In addition, cross-reactivity among viruses in the same family can lead to misdiagnoses. For example, past infection or vaccination with a flavivirus can lead to spurious ELISA or IF antibody results for other flaviviruses, and PRNT may be needed to establish etiology. Primary infection with EBV can lead to a false-positive CMV IgM and vice versa; thus antibody to EB nuclear antigen to confirm a primary EBV infection or CMV viral load in blood to assess CMV activity may be needed for an accurate diagnosis.

Molecular amplification methods have revolutionized diagnostic virology, especially for CNS disease. First applied to testing CSF for HSV encephalitis in 1990, PCR quickly replaced brain biopsy with culture as the method of choice (Rowley et al., 1990). CSF PCR is now the standard test for many viruses in CSF. Real-time PCR methods have reduced assay time and carryover contamination, made quantitation easier, and allowed rapid introduction of assays into laboratories capable of implementing homebrew assays. However, there are still few FDA-cleared test kits. That is changing as demand grows and companies see opportunities. Respiratory virus testing, especially for influenza, has motivated manufacturers to make simplified tests available to routine laboratories.

Despite the many advantages of molecular methods, it is important to remember that both false-positive and false-negative results occur. Due to its high sensitivity, PCR is susceptible to carryover contamination from amplified DNA or cross-contamination from positive clinical samples. In addition, latent viruses can reactivate without symptoms and mislead clinicians. EBV is carried for life in lymphocytes and in the presence of CSF lymphocytosis and immunosuppression, EBV DNA can be detected in CSF but is not linked to disease (Davies et al., 2005). Blood contamination of CSF can give rise to positive CSF PCR results that reflect viremia, not CNS infection. VZV can be detected in CSF in uncomplicated zoster with no CNS disease. HHV-6 can be found in high titer in the blood of persons without disease due to integration of the virus into the host cell chromosome of 0.5–1.0% of the population. Unfortunately, finding HHV-6 in the blood during an unrelated illness can mislead clinicians (Hubacek et al., 2007). Indeed, a rising titer of a virus in blood or CSF often has greater clinical relevance than a positive one at a single point in time.

Molecular testing can have other untoward effects. For example, the replacement of enterovirus culture of CSF by PCR has had the unanticipated consequence of eliminating detection of parechoviruses, which cause similar disease but require a separate PCR that is not widely available (Nix et al., 2008). Unappreciated genetic variability or viral genome mutations can lead to falsely negative results, as in a case of progressive multifocal leukoencephalopathy due to JC virus, and thus impair patient management (Landry et al., 2008).

Lack of standardization is a major issue, as few international quantitation standards exist against which to benchmark the sensitivity of an assay. In one multicenter study of HSV PCR assays, up to 50% of results for samples that contained 200–7000 copies of HSV/mL CSF were falsely negative (Schloss et al., 2003). In two international studies of transplant centers, CMV and EBV viral load copy numbers reported on the same samples varied by 100–1000-fold between laboratories (Pang et al., 2009; Preiksaitis et al., 2009). Without international standards, setting viral load cut-offs or reporting the number of DNA copies a test can detect is meaningless.

Viral diagnosis is an extremely dynamic and rapidly changing field, and with tremendous progress has come new challenges. With the increasing complexity of test options, the speed of methodologic change, and discovery of new viruses and therapies, it is imperative that clinicians and laboratory professionals consult the latest literature, work together, and communicate to optimize patient testing and result interpretation.

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