Diagnosis of Viral Infections

6

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Abstract

Accurate diagnosis of viral infections enhances the ability of the clinician to make decisions on appropriate treatment of patients, evaluate disease progression and prevent misuse of antibiotics. Knowledge of the pathogen involved also allow implementation of infection control and monitoring of success of antiviral treatments that may affect the prognosis of patients. Epidemiological data collected through accurate diagnostics play an important role in public health through identification and control of outbreaks, implementation of appropriate diagnostic tests, vaccination programs and treatment but also to recognize common and emerging pathogens in a community. It is key that the clinician have an understanding of appropriate specimens to send to the laboratory and the value of specific nucleic acid and serological testing for different viral pathogens. Molecular techniques have revolutionized viral diagnoses over the past decade and enhanced both the sensitivity and specificity of tests and the speed by which a diagnosis can be made and new tests be developed. The continued use of serology for viruses with a short viremia, or for chronic infections should however complement these tests. This chapter aims to provide an overview of the available tests, the principles of testing and appropriate tests to select for different viruses and syndromes. Also provided is a glimpse of new developments in diagnostics that may further enhance the capacity to make a conclusive diagnosis in the near future.

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6.1 Introduction

Human virus infections may affect all ages and may impact morbidity and mortality through acute, chronic, recurrent or lifelong infections. This may depend on the immune status of the patient and their ability to clear virus infection as well as the characteristics of the pathogen. The development of sensitive and specific methods for both the detection of viral nucleic acids and antiviral antibodies has greatly advanced our ability to make accurate diagnoses at different stages of the disease. Previously the extended periods needed for identification of viral etiologies; which greatly depended upon virus isolation techniques, meant that most viral diagnoses were of epidemiological value only [1–4].

Advances made in diagnostic techniques over the past decade have significantly improved the accuracy and timeliness of a viral diagnosis, which in turn can aid in patient management, disease control and positively impact the disease outcome [1–4]. Since the development of antiviral drugs and treatment options available for viral infections, clinicians are encouraged to seek viral laboratory diagnosis that can provide clinically useful information in diagnosis and management of patients. This required the focus of laboratories to shift to providing better, faster diagnosis, which has driven the development of new approaches to monitor viral infections and to support antiviral treatment through: quantitative viral loads, antiviral susceptibility testing, viral genotyping and, point-of-care testing. Despite the massive impact that molecular diagnostics has had on viral diagnosis, significant strides have been made in antigen detection and serological tests, in development of "rapid tests", for the direct detection of viral antigen in clinical specimens and detection of antibodies in convalescent or chronic infections. Laboratory controlled molecular and serological tests continue to have the advantage of superior sensitivity, specificity and differential diagnostic options in a controlled environment [1–4]. With the increase in sensitivity, specificity and diversity of virological diagnostic assays available, the clinician should work in collaboration with the virology laboratory to maximize the diagnostic potential of an appropriate clinical specimen. Understanding the relevance of the diagnostic test requested for specific viruses, at different ages and interpretation of a positive test, remains key in the clinical management of a patient [1–4].

The aim of this chapter is to provide an overview of diagnostic methodologies available for viral diagnosis rather than extensive technical details of each of the assays. It aims to provide an overview of options available for the clinician, from common assays to recent developments; the rationale for using each and how they could be successfully employed for better clinical management of patients.

6.2 Collecting and Sending Clinical Samples to the Laboratory

The most important factor influencing the accuracy of viral diagnostic results is the specimen. Whichever method is used in the laboratory, the results are largely dependent upon the right specimen type, taken at the right time and stored and transported correctly [5].

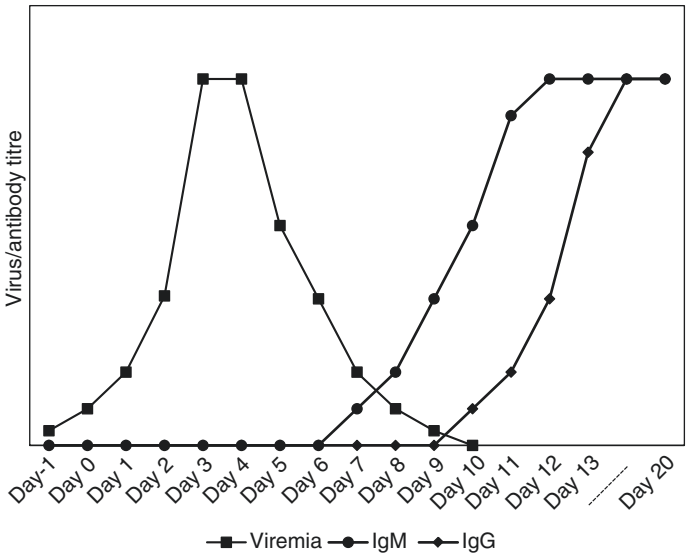

Fig. 6.1 Diagram indicating a typical acute virus infection showing the period that virus, IgM and IgG antibody can be detected (compiled from [4, 6, 8])

An understanding of the pathogenesis and epidemiology of the virus involved will help to identify the correct test and specimen type to collect. For acute viruses it is crucial to take the time since infection into consideration and whether the virus circulates commonly in the population and causes reinfections. Figure 6.1 depicts the typical period that acute viruses can be detected in blood and the time before IgM becomes visible and later IgG [4, 6]. For many acute viruses the viremia varies but may be relatively short and virus can only be detected in the first 10 days from the time that clinical symptoms became apparent, either in the blood or urine if it causes a systemic infection, such as arboviruses or measles; in the stool for enteric viruses such as rotavirus or poliovirus; in the site of infection such as the respiratory tract for respiratory viruses; or central nervous system for neuroinvasive infections. During this time virus specific tests such as virus isolation, antigen tests or molecular tests such as reverse transcription (RT) polymerase chain reaction (PCR) are appropriate. For viruses that are less common in the environment such as the arboviruses and childhood diseases prior to vaccination, IgM antibody tests can be requested after 7–10 days, but may not be detected in early specimens and are not appropriate for common viruses such as the respiratory viruses that may cause frequent reinfections. IgG antibody would only be used to diagnose acute infections if a paired serum is available 10–14 days apart and is not used for common viruses that may cause frequent reinfections. IgG testing may also be used to determine immune status following vaccines. Maternal antibody will interfere with IgG testing the first 4–6 months of life and therefore, for chronic diseases such as HIV transmission in children from HIV-infected mothers, DNA PCR testing is more suitable [4, 7, 8].

6.2.1 Type of Specimen

Specimens to be used for virus isolation and RTPCR should be kept below 4 °C (39.2 °F) and reach the laboratory within 72 hours to keep RNA intact. For enveloped single stranded RNA viruses such as RSV the success rate declines from 48 hours and all effort should be made to keep the specimen on ice from the time it is collected until it reaches the laboratory.

Blood: the usual required volume is between 2 and 10 ml depending on the patient's age, with the appropriate tube determined by the test required, and the appropriate blood component for the test (whole blood, plasma, serum). Anticoagulants such as heparin may inhibit PCR and EDTA tubes are preferred for molecular testing. For serology, clotted blood may be collected in SST (serum separation tubes) tubes that allow separation of red blood cells and serum through centrifugation. Virus isolation may be preferred from whole blood (EDTA) or serum (clotted blood) depending on the virus. *Swabs:* Swabs with a Dacron or rayon tip are preferred to ensure cells are collected and should be placed in viral transport medium that will preserve labile viruses for viral isolation and RTPCR. Washes or aspirates such as nasopharyngeal aspirates and other fluids such as saliva and urine should be placed in viral transport medium, although CSF is usually preferred undiluted. *Stool:* Obtain at least 4 g of stool and place in a sterile container. *Tissue*: Place in a sterile container with small amount of viral transport medium, for viral diagnosis. Specimens other than clotted blood must be kept at 4 °C (39.2 °F) and transported on ice to retain viability of the viruses and keep nucleic acids intact [7]. Table 6.1 summarizes the type of specimen and relevant tests available for viruses associated with different syndromes that may affect children (and adults).

6.3 Methods Used in Diagnostic Virology

6.3.1 Electron Microscopy

Although this is one of the oldest techniques it is not routinely used in diagnostic laboratories anymore. Electron microscopy (EM) is the only method available for directly visualizing the virus, and therefore has many applications beyond being purely diagnostic. The visualization of viruses with EM involves negative staining of the clinical specimen. Negative staining of the clinical sample is a relatively straightforward; inexpensive technique that would represent a "catch all" method of viral identification. EM could be particularly useful in identifying fastidious [87] or non-cultivable [88–90] virus in specimens, providing they have a high virus concentration with a sensitivity limit of approximately 10^6 viral particles per milliliter of specimen, making a negative result difficult to interpret [2]. While the sensitivity could be increased by ultracentrifugation or antibody-induced clumping, a further limitation is the lack of specificity, as EM can only identify up to the family level whereafter, other methods would have to be applied for a specific diagnosis [3].

Table 6.1 Specimen information for diagnostic virology; compiled from [1, 4, 9]

General syndrome	Agent	Specimen required	Diagnostic test options					Comments
			Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
					IgM	IgG		
Respiratory (pharyngitis, croup, bronchitis, pneumonia)	Adenovirus	Combination NP/OP swab; NPA, BAL	Yes	21 or Shell-vial	N/A	N/A	Yes [10, 11]	Virus has no specific seasonality and is detected all year round [10, 12, 13]. Shell vial tests followed by antibody staining allows detection in cells after 48–96 hours
	Coronavirus	Combination NP/OP swab; NPA, BAL	No	RL	N/A	N/A	Yes [14–16]	This includes emerging viruses SARS-CoV and MERS-CoV [17]
	Cytomegalovirus	Combination NP/OP swab; NPA, BAL, Blood	Yes	28	Yes	N/A	Yes [18]	CMV pneumonia in severely immunosuppressed HIV-positive patients and congenital infections [18]
	Enterovirus	Combination NP/OP swab; NPA, BAL	No	14	N/A	N/A	Yes [10, 12, 13, 19–21]	Virus has no specific seasonality and is detected all year round [10, 12, 13]

(continued)

Table 6.1 (continued)

General syndrome	Agent	Specimen required	Diagnostic test options					Comments
			Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
					IgM	IgG		
	Herpes simplex virus (HSV)	Combination NP/OP swab; NPA, BAL	No	1–7	Yes	N/A	Yes	HSV-1 has been associated with severe acute respiratory disease in severely immunocompromised patients [22]
	Human metapneumovirus	Combination NP/OP swab; NPA, BAL	No	2–21	N/A	N/A	Yes [10, 12, 15, 16, 23–25]	Virus not routinely cultured and IFA not available. Seasonality similar to that of RSV [10, 12, 13, 25].
	Influenza virus	Combination NP/OP swab; NPA, BAL	Yes	2–14	N/A	N/A	Yes [10, 24, 26, 27]	Antigen detection 40–90% sensitive. Seasonal, test for Influenza A and B. Subtyping for seasonal subtypes (H1N1pdm09/ H3N2) by specialist laboratories
	Parainfluenza virus (PIV)	Combination NP/OP swab; NPA, BAL	Yes	2–14	N/A	N/A	Yes	Of all the PIVs, PIV3 is the main contributor to respiratory disease with a seasonality in the spring and summer months, PIV1, 2 and 4 less common [12, 13, 28].

		Respiratory syncytial virus	Combination NP/OP Swab in VTM; NPA, BAL	Yes	2–21	N/A	N/A	Yes [10, 15, 27, 29–34]	Rapid antigen detection variable specificity; 90% sensitive [35, 36]; Strong seasonal trends in autumn-winter months in South Africa; winter in temperate climates in Northern hemisphere, rainy season in tropics [10, 12, 13].
		Rhinovirus	Nasopharyngeal (NP) aspirate (NPA); NP wash; or NP swab, Oropharyngeal swab (OP), Combination NP/OP swab	No	2–7	No	No	Yes [19, 37, 38]	Too many strains to type serologically. Virus has no specific seasonality and is detected all year round [10, 12, 13].
Exanthem	Maculopapular	Arboviruses	CSF if neurological; plasma/serum for febrile, VHF	No	RL	Yes	Rise in antibody levels: paired sera 10–14 days apart	Yes [39–44]	Viremia short, RTPCR in first 10 days only making acute serum for IgM serology important. Neurological cases detected in CSF. Specific to geographic region, mostly dengue, Zika. Cross reactivity for flaviviruses complicate confirmation by serum neutralization assays required

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Table 6.1 (continued)

General syndrome		Agent	Specimen required	Diagnostic test options					Comments
				Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
						IgM	IgG		
		Enterovirus	CSF when clinically relevant; OP or rectal swab	No	14	N/A	N/A	Yes [10, 12, 13, 19–21]	
		Human herpes virus 6 and 7	Serum	No	RL	Yes	No	Yes	Roseola agent
		Measles virus	Serum, urine, respiratory secreta or CSF depending on syndrome	Yes	RL	Yes	Yes	Yes	Difficult to grow; RTPCR during acute, IgM serology later in disease for diagnostic purposes with paired sera [1, 9]
		Parvovirus B19	Serum	No	No	Yes	ND	Yes [45–50]	Erythema infectious agent; IgM serology is often diagnostic, but may be positive for a prolonged period [1]
		Rubella virus	CSF when clinically relevant, serum, urine	No	>10	Yes	Yes	Yes	Recommended that paired sera be tested simultaneously for diagnostic purposes [1, 9]
	Vesicular	Herpes simplex virus	CSF when clinically relevant; Vesicle fluid, serum, EDTA	Yes	21	Yes	Yes	Yes [51, 52]	Serology rarely used for herpes simplex; IgM antibody used in selected cases [1]. Vesicle scrapings for direct IFA test [9]

		Varicella-zoster virus	CSF when clinically relevant; Vesicle fluid, serum, EDTA	Yes	21	Yes	Yes	Yes [52]	Vesicle scrapings for direct IFA test
CNS (Aseptic meningitis and encephalitis)		Arboviruses	CSF when clinically relevant and serum	No	No	Yes	Yes	Yes	Viremia brief, negative molecular test should be followed up with IgM serology with acute serum
		Dengue	CSF when clinically relevant, serum	Yes	RL	Yes [53]	Yes	Yes [54–57]	According to CDC; 80% seropositive at 6 days. Viremia brief, negative molecular test should be followed up with IgM serology with acute serum
		Cytomegalovirus	CSF when clinically relevant	No	RL	Yes	Yes	Yes [51]	Immunocompromised patients; newborns
		Enterovirus	CSF when clinically relevant	No	3	No	No	Yes [58–60]	Most common viral cause of meningoencephalitis
		Epstein-Barr virus	CSF	No	No	Yes	Yes	Yes [51]	Neurological cases confirmed on CSF, positive on serum need to be interpreted with caution due to reactivation
		Hantavirus	Serum, CSF when clinically relevant	No	RL	Yes	ND	RL	Diagnosis by presence of IgM antibody, Dependant on geographic location (Americas/Europe)

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Table 6.1 (continued)

General syndrome	Agent	Specimen required	Diagnostic test options					Comments
			Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
					IgM	IgG		
	Herpes simplex virus	CSF when clinically relevant, serum	Yes	21	Yes	Yes	Yes [51, 61]	Culture of CSF has very low sensitivity; RTPCR on CSF most reliable
	Measles virus	CSF when clinically relevant, serum	Yes	21	Yes	Yes	Yes	Culture of virus usually very successful. IgM serology diagnostic and RTPCR
	Mumps virus	CSF when clinically relevant, urine	No	21	Yes [62]	Yes	Yes [63, 64]	RTPCR/IgM ELISA may allow diagnosis
	Rabies virus	Saliva, nuchal skin biopsy, CSF (pre-mortem); brain biopsy (postmortem)	Not WHO endorsed	2–3	N/A	Neutralizing antibodies on CSF only	(WHO), no commercial tests	Antigen detection (FAT) on tissue (brain); RTPCR tissue; CSF, saliva; Serology only on unvaccinated individual
	Varicella-zoster virus	CSF when clinically relevant, vesicular/skin swab	Yes	3–21 days [1]	Yes	Yes	Yes [51, 52]	

	West Nile virus	CSF when clinically relevant, EDTA blood; serum	Yes	RL	Yes	Yes	Yes [39, 65–67]	Viremia brief, RTPCR on CSF or EDTA blood; if negative follow up with IgM serology with acute serum. Cross reaction of flaviviruses require confirmation by serum neutralization assays
Infectious mononucleosis	Epstein-Barr virus	Serum	No	No	Yes	Yes	Yes [51]	PCR on blood most common but correlate with clinical presentation. Serology for infection status only
Hepatitis	Hepatitis A virus	Serum	No	No	Yes	ND	Yes [68–70]	Diagnosis by presence of IgM antibody/PCR
	Hepatitis B virus	Serum	Yes	No	Yes	ND	Yes	IgM to surface or core antigen confirms diagnosis; HBV viral load by PCR used for disease progression and clinical management
	Hepatitis C virus	Serum	No	No	Yes	ND	Yes	Serology and PCR confirms infection; viral load by PCR used for management
	Hepatitis D virus	Serum	No	No	Yes	No	No	Virus not routinely cultured
	Hepatitis E virus	Serum, stool	No	No	Yes [71]	Yes	Yes [72, 73]	Virus not routinely cultured

(continued)

Table 6.1 (continued)

General syndrome	Agent	Specimen required	Diagnostic test options					Comments
			Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
					IgM	IgG		
Immunodeficiency virus	HIV1/2	EDTA and serum	Yes	15	Yes 5–28	ND?	Yes	Antibody confirms infection in adults; DNA PCR for children <18 months due to passively acquired maternal antibody. Viral load by realtime PCR used for disease progression and clinical management
Gastroenteritis	Adenovirus	Rectal swab, stool	Yes	10	No	No	Yes [74–77]	Adenovirus 40 and 41 implicated in pediatric gastroenteritis
	Astrovirus	Stool	Yes	RL	No	No	Yes	Diagnosis by electron microscopy
	Norovirus or Norwalk	Stool	No	RL	No	No	Yes [78–82]	One of the major causes of acute gastroenteritis in communities or cruise ships
	Rotavirus	Stool	Yes	RL	No	No	Yes [76, 77, 83, 84]	Rapid assay are usually reliable, seasonal

Congenital infections	Cytomegalovirus	EDTA, serum, amniotic fluid, cord blood	Yes	2	Yes	N/A	Yes [51]	Presence of CMV IgM in cord blood is indicative of congenital infections; PCR and culture on dried blood spots, blood, saliva or urine in newborn < 3 weeks; retrospective, should be done on stored dried blood spots taken after birth [8]
	Enterovirus	CSF, serum, cord blood	No	3	Yes	N/A	Yes [58–60]	Cord blood most appropriate specimen in congenital infections
	Herpes simplex virus	CSF, dermal, vesicle swab, tissue biopsy, amniotic fluid, cord blood	Yes	1	Yes	N/A	Yes [51]	Presence to HSV IgM in cord blood indicative of congenital infections
	Parvovirus B19	Synovial fluid, amniotic fluid, plasma	No	No	Yes	ND	Yes [45–50]	
	Rubella virus	CSF, serum	No	>10	Yes	N/A	Yes	IgM for Rubella should be assayed using serum from infants up to 6 months of age IgG; could be false positive due to maternal antibodies

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Table 6.1 (continued)

General syndrome	Agent	Specimen required	Diagnostic test options					Comments
			Commercial Rapid antigen detection test (RADT) and IFA available	Virus Isolation period in days	Diagnostic ELISA		PCR: Commercially or in-house assays	
					IgM	IgG		
	Zika virus	CSF, EDTA blood, serum. Pregnant mother/infant	No	RL	Yes	N/A	Yes	RTPCR recommended tests; IgM commercial tests need to be confirmed by PRNT by a reference laboratory for arboviruses due to cross reaction with dengue and other flaviviruses, depending on geographic relevance or travel history (CDC testing regiment [85, 86])

CSF cerebrospinal fluid, *ELISA* enzyme-linked immunosorbent assay, *PCR* polymerase chain reaction, *EDTA* ethylenediaminetetraacetic acid (purple top tube), *NP* nasopharyngeal, *OP* oropharyngeal, *NPA* nasopharyngeal aspirate, *BAL* bronchoalveolar lavage, *IFA* immunofluorescent antibody, *RL* indicates specialist or research laboratory only, *ND* not done, *N/A* Not applicable

Although the major advantage of EM is the speed with which a result could be obtained (30 min), the high cost of the instrument and specialized training and expertize needed, coupled with the lack of sensitivity and specificity, does not make this a viable option for routine diagnostics [4, 8].

6.3.2 Histology/Cytology

Direct microscopy of stained histology or cytology specimens may, in some instances, give the first indication of viral involvement that involves cellular changes. For viruses such as CMV, VZV, HPV, BK and B19, specific cytological changes can be confirmed through staining for specific antigen or genome sequences, using antibody or nucleic acid probes. Specific PCR amplification techniques may outperform these techniques in sensitivity, although detection of antigen in tissue is highly specific [4, 8].

6.3.3 Virus Isolation

Viral tissue culture was traditionally the "gold standard" used for diagnosing virus infections [91]. However, in the last 10 years molecular techniques have become routine. Virus isolation needs to remain an important part of viral diagnostics in order to maintain a source for analyzing, not only genotypic changes, but also phenotypic changes in virus populations for vaccine relevance and epidemiology. This allows identification of changes in antigenicity, pathogenicity and viral characteristics to update vaccines, such as the influenza vaccine, to match circulating strains [92]. Quality of the specimen, the time that it takes to reach the laboratory and transport under cold chain will determine the success of virus isolation. Detection of viruses in cell culture requires a considerable expertise and is performed by microscope examination, looking for degenerative morphological changes in the cell monolayer. This is called the cytopathic effect (CPE). Not all viruses grow in all cell types or produce CPE and further antigen or nucleic detection methods are required to correctly identify the specific virus involved. Clinical specimens are usually inoculated onto several cell lines to provide an optimum environment for a range of viruses (Table 6.2) [93].

An adaption of traditional viral culture formats has been developed, which allows for more rapid detection of viruses, especially for viruses which are known to grow slowly in conventional cell culture. This is achieved by inoculating the specimen onto a microscope slide and centrifugation of the culture to enhance the infection rate (Shell vial assays). The enhanced detection rate may result from better contact between cells in the specimen and the cell culture, thus allowing for earlier and more extensive infection of the cell lines, as well as through the use of fluorescent-labeled (e.g. FITC) monoclonal antibodies directed to the viral antigen [93]. Nevertheless, most culture methods lack sensitivity and specificity relative to PCR. It remains, however, a catchall method of choice if the virus in question can be cultured [92].

Table 6.2 List of viruses commonly isolated in clinical laboratories; compiled from [4]

Virus	Rate of growth [1, 94][a]	Type of CPE that can be detected [94]	Most permissive cell line[b]
RNA viruses			
Enteroviruses	2–8	Retractile angular or tear-shaped cells	PMK
Rhinoviruses	4–10	Retractile rounding of cells	HDF
Influenza viruses	2–14	Swollen vacuolated cells	PMK
RSV	2–21	Syncytia seen only in Hep-2 cells	HEp-2
DNA viruses			
Adenoviruses	1–21	Aggregation and rounding of cells in grape-like structures	HEp-2
HSV	1–7	Retractile rounded cells	A549
VZV	5–10	Foci of enlarged cells	HDF
CMV	5–28	Small foci of enlarged cells	HDF

[a]Time in days needed for CPE to develop, depending on the initial viral load in the sample, the higher the viral load the quicker CPE will be detected
[b]*PMK* Primary monkey kidney, *HDF* human dermal fibroblasts, *HEp-2* human epithelial type-2, *A549* adenocarcinoma human alveolar basal epithelial cells

6.3.4 Nucleic Acid Detection Methods

Viruses can be detected directly in clinical samples using highly specific nucleic acid primers and probes that are complementary in sequence to RNA viruses, using RT-PCR or for DNA viruses, directly by PCR. Over the past 10 years, nucleic acid amplification tests have been developed for the major viruses of public health concern and have become the new benchmark for viral diagnoses. The published sensitivities and specificities are usually nearly 100% when compared with cell culture or antigen assays [92, 95–97]. In fact studies that have compared molecular assays, with tissue culture assays, have demonstrated significantly increased sensitivity, of up to 30% [92, 95, 96, 98, 99].

The development of real-time PCR, that incorporates the use of specific florescent labeled probes, has created the ability to monitor the DNA amplification process as it happens, or in "real-time" on a dedicated instrument that is capable of collecting the fluorescent data from every PCR cycle. The accumulation of the measured fluorescence at the end of every PCR cycle is plotted and displayed as a sigmoidal curve and when the data is analyzed a cycle threshold (Ct) value is assigned to each target's amplification when it is first detected. The Ct is the point at which the amplicons' fluorescence exceeds that of the background and this is indirectly proportionate to the initial concentration of the target DNA in the sample i.e. the higher the concentration in the initial sample the lower the Ct value will be [100–102].

Comparative studies have revealed that the detection of respiratory viruses using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays is substantially more sensitive than using conventional methods such as viral culture and

immunofluorescence assays (IFA) [92, 103, 104]. Furthermore, compared to conventional PCR and other real-time methods, multiplex rRT-PCR has the advantage of permitting simultaneous amplification of several viruses in a single reaction [16, 103, 104]. This facilitates cost-effective diagnosis, enabling the detection of multiple viruses in a single clinical specimen. Amplification of several viruses together may however sacrifice sensitivity of individual assays and much effort has gone into identifying ways of increasing sensitivity.

6.3.4.1 Multiplex PCR Assays

Multiplex PCR assays are now frequently used to detect the presence of a range of viruses involved in specific syndromes such as respiratory infections e.g. influenza virus (INF) A and B; [20, 95, 105–107], parainfluenza viruses (PIV) types 1, 2, 3 and 4 [19, 20, 95, 105, 107, 108]; human respiratory syncytial virus (RSV) [20, 105–108], human metapneumovirus (hMPV) [20, 105, 107], human rhinoviruses (RV) [19, 20, 105, 107], human coronaviruses (hCoV-229E, hCoV-OC43) and Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) [20, 107], human enteroviruses (EV) [19, 20] and adenoviruses (AdV) [108]. Disadvantages, include higher start-up costs, higher reagent costs, and extensive and specific training for specialist laboratories and specialized equipment to run them [92].

The diversity, fastidious nature, short viremia periods of some pathogens that may cause the infection and lack of available diagnostic tests, can severely hamper the ability to identify etiologies to different clinical syndromes [81]. Several multiplex platforms have been developed either as a set of duplex assays, in-house and commercial [20, 104, 109] and multiplex systems that require specialized equipment to read and are now available for a range of syndromes [20, 104, 109]. The limiting factor has been sensitivity and most assays require PCR amplification before detecting products through a number of platforms. These include Mass-Tag PCR [110], microarray platforms [111], macro arrays [39], microbead based methods [20] and Taq-Man array cards [112–115]. Currently TaqMan array cards are increasing in popularity due to the ease with which these can be adapted for specific purposes. These assays have application in diagnosis of single cases or as part of epidemiological studies to describe the etiologies of specific syndromes. TaqMan array card (TAC; Life Technologies, Foster City, CA, USA) assays have been developed and used with success for several syndromes such as respiratory disease [63], enteric disease [70] and neonatal sepsis [81]. Once developed, TaqMan array cards are stable at 4 °C (39.2 °F) for 2 years and can be shipped at ambient temperature [116]. The TAC assay is a 384-well microfluidic array which consists of identical arrays in eight individual microfluidic channels, each of which can be loaded with nucleic acid extract from a clinical specimen or positive control [63, 70, 81, 116, 117]. The individual channels consist of 48 wells, each of which contains singleplex qPCR reactions targeting a different pathogen. Thus eight specimens are assayed per TAC card, and can simultaneously detect up to 48 pathogens per specimen. This makes the TAC assay popular for the following reasons: (1) minimal specimen volume required; (2) reduction in cross contamination of specimens due to the closed system format; (3) the ability to tailor the panel of

pathogens detected as required; (4) proven efficacy of this technology in pathogen detection for similar studies; (5) and simple to use format [63, 70, 81, 116, 117].

6.3.4.2 Future Trends

Multiplex methods are becoming more common in routine diagnostic laboratories. However, most of the large scale methods described above are predominantly research based and used in epidemiological studies or in specialist laboratories, rather than routinely. Next generation sequencing methods, that make use of deep sequencing of all nucleic acids present in a sample, are currently mostly used for pathogen discovery or in specialist laboratories to detect outbreaks. In general amplification steps are still needed before this can be used on clinical specimens. In addition these techniques are too expensive to run on a large scale, in routine diagnostic laboratories. Even though these techniques are becoming more affordable they generate significant amounts of data that require both trained bioinformaticians to interpret the outputs and large computational systems. Nevertheless, development of automated systems for identifying viruses directly in clinical specimens, may in future make these techniques more accessible for routine diagnostics [118–120].

6.3.5 Application of Molecular Virology Diagnostics in Clinical Management of Patients

6.3.5.1 Qualitative vs. Quantitative PCR

The increased sensitivity that the development of molecular assays have highlighted, is that while there are advantages in identifying new viruses associated with disease, such as the respiratory viruses described in the last decade (including hMPV [23], hCoV NL63 [14] and HKU1) [121], it has also revealed flaws that make interpreting such a positive PCR result problematic. Recent literature has shown that specifically in the case of RV [27, 37, 38] that the virus was detected in asymptomatic as well as symptomatic patients. Due to the increased sensitivity of molecular assays it is possible to detect the presence of a virus at a low genome copy number, which may represent the pre- or post-syndromic phase of a viral infection, redefining the nature of viral disease and the clinical interpretation thereof [27, 38]. Interpretation of qualitative and quantitative PCR results as well as the application of the appropriate choice would require a close liaison with the virological laboratory.

Qualitative detection in specimens that are normally virus free: A good example of this is the diagnosis of viral or aseptic encephalitis, in which testing CSF for HSV, CMV, VZV or enteroviruses are diagnostic [122]. Qualitative PCR offers significant advantages in terms of speed, especially with the development of the point-of-care testing. Early diagnosis and treatment of CNS infections has been proven to improve the prognosis [117] and reduce unnecessary treatment and hospitalization [123]. Viruses that only exhibit low-levels of virus shedding in the absence of symptoms such as viral gastroenteritis, caused by rotavirus or norovirus could be detected in stool samples [81, 82].

Quantitative viral loads: Assays that can quantify the amount of virus in infected patients have proven to be the most valuable tool in the management of chronic viral infections. For many persistent viral infections, with transient low-level viremia, the onset of symptoms is associated with a spike in viral replication and thus a higher viral load, allowing the prediction of disease onset [8]. This allows for better clinical management of the patient, as the clinician can monitor the progression of the disease, the success of treatment, the emergence of drug resistance and understanding the pathogenesis of a particular virus of which HIV-1 and 2 [124, 125], CMV [116, 126], EBV [127], HBV [128, 129] and HCV [124] are but a few.

6.3.5.2 Antiviral Resistance

As the availability of antiviral drugs increases, more emphasis is placed on assays to determine the causes of treatment failure, of which antiviral resistance is one possible outcome [8]. The emergence of antiviral resistance has been documented for virtually all antiviral compounds, with the specific viral mutations associated with resistance becoming better understood [8, 125, 130–132]. Laboratory assays to determine drug resistance fall into two major categories:

Phenotypic assays: Phenotypic assays have largely been replaced by molecular based genotypic assays, however, they remain the gold standard for determining drug efficacy and susceptibility, as the concentration of the drug required to inhibit viral replication can be calculated. HIV is the best example [130, 131, 133, 134]. Phenotypic assays have the added advantage of giving a complete overview of all mutations observed. However, it is an expensive and laborious technique, of which the success will greatly depend on the level of training of staff and whether or not the specific virus culture-adapted strains are available [8].

Genotypic assays: While the development of RT-PCR as a genotypic assay, focusing on specific areas on the virus genome, have the added advantage of being rapid, relatively inexpensive and semi-quantitative (single point mutation assays, and allelic discrimination assays), it is difficult to interpret a single point mutation without all the required information that a phenotypic assay would provide [8]. The development of new automated sequencing methods have enabled the study of the genetic basis of drug resistance and made the assessment of virus isolates, with reduced drug susceptibility, more accessible [8]. The use of sequence based methods for testing for antiviral resistance have also have become routine in viral diagnostics, especially for HIV [132, 135–137] and HBV [138–140]. The biggest drawback of this technique, other than the expense, is the downstream analysis of sequencing data that is generated. Sequencing editing and interpretation is required, and in the case of HIV, the identification of resistance is dependent upon the recognition of specific sequence patterns on the software system used [141].

6.3.6 Serology

Serological techniques can either be targeted at the antigen, during the acute phase of infection, or to virus specific antibody later in infection. While virus specific antigen may only be detected in the first 10 days of acute infections, IgM antibody

is detected within 7–14 days following infection and may remain for a month or more in the patient's blood after the infection was cleared. Therefore, IgM can be used to detect a recent infection. IgG antibody is detected after 10–14 days of infection and can be present for life. Sero-conversion to IgG is measured with paired sera taken during the acute and the convalescent phase, 10–14 days apart. A significant rise in antibody of fourfold increase is seen as a positive reaction and new infection, while a single IgG positive test may reflect infection any time in the past. In pediatric infections, maternal immunity needs to be taken into consideration in the first 6 months of life and may only be cleared by 18 months, and may therefore, interfere with serological diagnosis in infants. Assays for detection of IgM or IgG are usually qualitative, since the presence or absence of antibody is enough to make a diagnosis. However, when a rise in antibody has to be detected, the test needs to be quantitative in order to detect an increase in antibody from the first to the second specimen. Antibody titre is measured as the reciprocal of the highest serum dilution where a positive reaction can still be detected. For example, a titre of 32 indicates that positive antibody binding could be detected in serum diluted up to 1 in 32, but not beyond that. Serological techniques are easily automated and play an important part in routine diagnostic laboratories. They have an important role in diagnosing acute and chronic infections and are useful for development of rapid tests and should, in addition, complement molecular techniques where clinically relevant [8, 142].

6.3.6.1 Immunofluorescence Assay (IFA)

These assays allow for the rapid detection of antigen and can be applied directly on clinical samples such as nasopharyngeal aspirates or on tissue culture or tissue from biopsy specimens, such as brain tissue for rabies virus. IFA is quick and convenient for individual specimens but requires a skilled operator and is not as easy to scale up and is not as sensitive as molecular techniques for viral detection. It is, however, relatively cheap and a popular choice, for this reason, in identification of viral infections.

Direct IFA detects virus in infected specimens or tissue using commercially available antibodies labelled with a florescent marker, while indirect IFA detects antibody in the patient sera by binding to the antigen in virus infected tissue cultured cells. A secondary anti-human IgG or IgM antibody is then used to detect the patient's bound antibodies. Direct IFA is frequently used for respiratory virus antigen detection in respiratory secretions (RSV, influenza, PIV1–3) while indirect IFA for IgG or IgM antibody detection is used to detect infections such as EBV or VZV, amongst others [4, 8, 142, 143].

6.3.6.2 Enzyme-Linked Immunoassay for Antibody Detection (ELISA)

ELISAs are the most commonly used antibody or antigen detection assays since they have a high throughput, are rapid, are easily automated and are objective since the output can be read using a spectrophotometer. ELISA works on the principle of detecting antibody in patient sera through a reaction where antigen is bound to the surface of a micro-titre plate, the patient serum added to bind to the antigen and any

bound antibody is then detected through addition of a secondary anti-human antibody coupled to an enzyme. Addition of a substrate to the enzyme linked antigen antibody complex results in a colour change which will induce a positive reaction. The assay can be adjusted for IgG or IgM through addition of the anti-human antibody. Antigen detection ELISA is performed by coating the solid phase with antibody to detect the antigen in the patient sera in order to reveal the detection antibody complex.

ELISA is frequently used for detection of IgG or IgM antibodies to rubella, measles, mumps, HIV, Hepatitis A and arboviruses such as West Nile virus, Zika virus, dengue or JEV. Although ELISA can have a very high sensitivity and specificity, some viral families may cross react and for exact identification of viruses such as the flaviviruses, neutralization assays are needed for confirmation [6, 142].

6.3.6.3 Neutralization Assays

Virus neutralization assays are highly specific assays testing for neutralizing antibodies and are also used to confirm results of other serological assays, such as ELISA, which are known to cross-react between different viruses of the same family e.g. the Flaviviruses (Zika virus, dengue and West Nile virus). It can also be used to determine if a vaccine would provide protection e.g. to detect antigenic drift in the neutralizing epitopes of the annual influenza vaccine. Antigen is mixed with dilutions of antibody and the inhibition of CPE observed through inoculation on a tissue monolayer. The inhibition effect can either be read through observation of CPE, or through overlay of agar which allows plaque formation for plaque reduction neutralization assays (PRNT). Micro-neutralization assays can also be read through ELISA methods, which help to automate the process and reduce the test run time before infected cells can be detected. These techniques are labour intensive and not routinely done by diagnostic laboratories, but rather by reference or specialist laboratories [6, 142, 144].

6.3.6.4 Other Serological Techniques

Several further formats of serological techniques exist that are used for different purposes. The **hemagglutination inhibition assay (HAI)** test detects antibodies to viruses that have a hemagglutinin antigen. These include rubella, Influenza and the flaviviruses. The test is still routinely used in reference laboratories to identify especially, cross reactive viruses such as the flaviviruses, before confirming specific viruses by neutralization assays or to investigate influenza antigenic variation relative to sera raised against the vaccine. Due to cross reactivity and requirement for fresh red blood cells, it is less commonly employed in routine laboratories. **The Western blot technique** is still used for confirmation of HIV and HCV, and it is based on the principle of transferring specific viral proteins separated on a gel or blotting paper, followed by binding to patient serum and detection with an anti-human enzyme labeled antibody and substrate. It is very specific but sensitivity may vary. Antigen detection methods allow for the development of rapid antigen or antibody tests and allow for bedside diagnosis. However, variable sensitivity and specificity determine the value of these tests [142].

6.3.6.5 Future Trends in Serology

Following the trend in molecular diagnostics, development of multiplex serological assays, that cover a range of viral antigens associated with specific syndromes, will significantly improve the diagnostic capacity of laboratories. Methods that use multiplex microsphere-based suspension immuno assays (SIAs) for the simultaneous detection of IgG antibodies against a range of viruses, enables development of syndrome or application specific tests. An example would be a B19, CMV and *T. gondii* combination SIAs multiplex for rapid antibody screening during pregnancy [145]. These assays bind a number of antigens through antibody to a microsphere. They are then incubated with the patient sera before being visualized with a labeled anti-human IgM antibody. Similar tests have been described for arbovirus screening in the Northern hemisphere [146]. Multiplex formats, based on protein arrays, have also been developed to detect a range of viruses. For these assays the antigens are fixed to a solid phase microchip slide and tested against patient anti-serum and fluorescent labeled anti-human antibodies used for detection. The position on the chip identifies the pathogen involved. These arrays may be based on peptides synthesized from pathogen sequence [147] recombinant proteins, [148] or inactivated virus antigens [149]. These techniques are not yet widely available in routine diagnostic laboratories but developed in specialist and research laboratories. However, the automation possibilities will very likely improve their accessible in the future.

6.3.7 Quality Assurance and Control

The ability of the laboratory to provide accurate diagnostic results is essential for effective clinical management of patients, solving of outbreaks and for responsible decision making [150]. Therefore monitoring ongoing quality assurance (QA) and improvement in all aspects of the laboratory is crucial. This involves the managing and monitoring of all services and processes related to releasing a diagnostic results [4, 150]. Processes that should be monitored relate to the pre-analytical phase *i.e.* specimen transport, collection and storage; the analytical phase *i.e.* testing and monitoring of the laboratory procedures and environment as well as the post analytical phase *i.e.* of result reporting and result interpretation [4, 150]. QA ensures annual assessment of staff competency, calibration and servicing of equipment as well as the quality of diagnostic tests. Clinical laboratories are strictly regulated by appointed agencies and are audited according to specific standards set forth by the International Organization for Standardization (ISO) and the International Electrotechnical Commission (IEC) [4, 150].

The primary quality control (QC) concern in a molecular laboratory is the specimen and nucleic acid quality or integrity, assay sensitivity and specificity, as well as the false positive tests because of PCR contamination. The RNA and DNA integrity can be insured through use of RNase and DNase free reagents and consumables, in addition to handling specimens on ice. While, PCR contamination can be avoided through physical organization of the laboratory and workflow, separating work areas and equipment; relevant PCR controls should be included in each

run to ensure correct interpretation of the results [4, 8, 150]. The use of uracil N-glycosylase (UNG) in PCR reactions provides for chemical control for carry over contamination [151].

It is vital that when PCR diagnostics are undertaken, every effort is made to minimize contamination and that these assays are tested in a laboratory environment in which staff are well trained and competent for this type of work [8]. Using an accredited laboratory ensures the diagnostic findings are reliable.

6.4 Conclusion

Molecular and serological techniques should be used in a complimentary fashion for the diagnoses of virus infections. Knowledge of the stage of infection, viral pathogenesis and epidemiology help to make decisions regarding the correct test to choose for appropriate diagnosis. Advances in specificity and sensitivity and capacity to test for a range of viruses through multiple platforms make accurate diagnosis and rapid identification of circulating viruses for real-time clinical relevant data more feasible today. Virus isolation and collaboration with specialist laboratories make newer techniques for identification of emerging and re-emerging viruses possible. Quality of specimens, patient clinical history and presentation and close collaboration between the clinician, pathologist and laboratory remain key for useful diagnostic data for improved patient management.

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