

Diagnostic Approaches

Katie Jeffery¹ and Emma Aarons²

¹*Department of Microbiology, John Radcliffe Hospital, Oxford, UK*

²*Centre for Virology (Bloomsbury), University College London, London, UK*

INTRODUCTION

Human virus infections may affect all ages and assume any degree of severity. They may be acute or chronic, be recurrent or elicit lifelong immunity. They are acquired through various routes via contact with humans, animals or the environment. They present as various syndromes involving fever, rash, arthralgia/myalgia, respiratory or gastrointestinal disorders and occasionally serious organ malfunction with deaths from pneumonia, cardiac, liver or kidney failure or encephalitis. They have to be rapidly distinguished from bacteriological and other infectious and non-infectious diagnoses if the appropriate clinical management is to be given.

Host factors are crucial to the outcome of virus infections. For any virus infection, age may be critical to determining outcome, those at extremes of age being more vulnerable as a consequence of lack of immunocompetence, inexperience of vaccination and waning of immunity. For some infections, gender and race may confer advantages or disadvantages, but malnutrition, pre-existing organ damage and social neglect are always potentially disadvantageous. Thus, in assessing prognosis and deciding on the investigation, management and treatment of virological infections, the individual patient must be carefully considered in their medical and social context. Any natural tendency towards spontaneous immune-mediated clearance of a virus is likely to be compromised if factors such as these are unfavourable.

A substantial part of clinical virology is taken up with the investigation and treatment of patients either constitutionally or iatrogenically immunosuppressed, or suffering from an existing immunocompromising infection, such as

human immunodeficiency virus (HIV). These patients often need pre-emptive and continuing investigation if intensive treatment for other conditions is not to be nullified by overwhelming virological and other opportunistic infections. The management of these patients must be planned and rigorous, and will differ from that of other virological patients.

Because virus infections are contagious, diagnosis cannot be confined to a consideration of the individual patient. Two questions may be crucial: where did the infection originate? And who may contract it next? Each question clearly gives rise to the potential for wider investigation and possible action to protect contacts through behaviour modification, isolation or prophylaxis with drugs or vaccines. If the infection is sufficiently contagious or is life-threatening, more extensive public health measures may be required and the clinical diagnosticians must not lose sight of the possible implications of their conclusions for the wider community.

Clinical virology in the 1980s was characterized by the widespread use of enzyme-linked immunosorbent assay (ELISA) technology, and in the 1990s by the entry into routine diagnostic use of molecular methods for virus detection. During the early years of the twenty-first century real-time polymerase chain reaction (PCR) and virus quantification have come of age, alongside increasing automation of molecular diagnostics. Concurrently, the emphasis and priorities of diagnostic virology laboratories have shifted. This is in response to the availability of rapid diagnostic methods, the identification of new viruses many of which are non- or poorly cultivable, the increasing availability of effective antiviral agents, the emergence of antiviral resistance, the increasing number

of immunocompromised patients in whom opportunistic viral infections are life-threatening, and the cost pressures on pathology services.

This chapter will provide, firstly, an overview of diagnostic techniques set against this background and presented in order of historical development. Secondly, it will highlight the ways in which these techniques may be applied to arrive at accurate diagnosis thereby facilitating effective management of virus infections, including prevention of their onward spread.

ELECTRON MICROSCOPY

Electron microscopy (EM) is the only technique available for directly visualizing viruses, and therefore has many applications beyond purely diagnostic ones. With the advent of alternative diagnostic methods, EM retains a limited role in the clinical setting for the diagnosis of viral gastroenteritis and examination of skin lesions for herpes and pox viruses.

Preparation of specimens for EM and the technique of negative staining are straightforward and quick, and the method is a 'catch-all' approach to detecting viruses. However, it has a limit of sensitivity of approximately 10^6 viral particles per millilitre of fluid, making negative results unreliable. Vast numbers of virions are present during acute skin and gastrointestinal disease and a diagnosis is easily made, but later in the course of infection viral shedding is reduced below the level of detection. Although sensitivity can be enhanced by antibody-induced clumping of virus (immune EM) or ultracentrifugation, it is unrealistic to undertake these methods routinely. The advantages and disadvantages of EM are summarized in Table 1.1.

The survival of EM within the routine clinical virology laboratory hinges on the emergence of alternative, more sensitive methods of diagnosis. Many centres now use latex agglutination for rotavirus diagnosis, and PCR is more sensitive than EM for detection of herpesviruses in vesicular fluid (Beards *et al.*, 1998) and for the detection of noroviruses (previously called Norwalk-like

viruses) (O'Neill *et al.*, 2001). Thus, the future of EM in clinical virology is in some doubt. However, one of the first indications for EM was for the rapid diagnosis of smallpox and, in the era of bioterrorism, EM may continue to play a role in specialist centres in the event of a bioterrorist attack.

HISTOLOGY/CYTOLOGY

Direct microscopic examination of stained histology or cytology specimens can on occasion provide the first indication that a virus may be responsible for a pathological process, for example the intranuclear (early) or basophilic (late) inclusions seen in interstitial nephritis in renal transplant biopsies due to BK virus, changes in cervical cytology seen in association with human papilloma virus (HPV) and the nuclear inclusions seen in erythroid precursor cells in Parvovirus B19 infection. Moreover, the particular viral aetiology can be confirmed by specific antigen/genome staining using labelled antibody or *in situ* hybridization techniques (see below).

VIRUS ISOLATION

Many of the advances in clinical virology have come about because of the ability to grow viruses in the laboratory. Historically, viruses were propagated in laboratory animals and embryonated eggs, but most virus-isolation techniques now rely on cultured cells. With appropriate specimens and optimal cell lines, this technique can be highly sensitive and specific, with a presumptive diagnosis made on the basis of a characteristic cytopathic effect (CPE). The particular diagnosis can then be confirmed by haemadsorption (certain viruses, influenza and measles for example, cause adherence of erythrocytes to infected cells in a monolayer because the viral antigens expressed include a haemagglutinin) or by immunofluorescence (IF) using a virus-specific antibody labelled with a fluorescent dye. The judicious selection of two or three cell lines, such as a monkey kidney line, a human continuous cell line and a human fibroblast line will allow the detection of the majority of cultivable viruses of clinical importance, such as herpes simplex virus (HSV), Varicella zoster virus (VZV), cytomegalovirus (CMV), enteroviruses, respiratory syncytial virus (RSV), adenovirus, parainfluenza viruses and influenza viruses. In addition, the ability to grow virus from a clinical specimen demonstrates the presence of viable virus (albeit viable within the chosen cell line)—this is not necessarily the case with detection of a viral antigen or genome. For example, following initiation of antiviral therapy for genital herpes, HSV antigen can be detected from serial genital swabs for longer than by virus

Table 1.1 Electron microscopy

Advantages	Disadvantages
'Catch-all'	Requires skilled staff
Detects unculturable viruses	Large capital outlay
Economical running costs	Poor sensitivity
Adaptable, for example immunolectron microscopy confirms cytopathic effect	
Rapid (same day)	

propagation in cell culture. This implies that antigen is persisting in the absence of viral replication and underlines the importance of correct interpretation of laboratory results. However, failure to isolate a virus does not guarantee that the virus is not present. Virus isolation has also been shown to be diagnostically less sensitive than molecular amplification methods such as PCR for HSV and several other viruses (see below), for example for the diagnosis of herpes simplex encephalitis.

The benefits of virus isolation (Table 1.2) include: the ability to undertake further characterization of the isolate, such as drug susceptibility (see later) or phenotyping; and the identification of previously unrecognized viruses, for example human metapneumovirus (van den Hoogen *et al.*, 2001), severe acute respiratory syndrome (SARS)-associated coronaviruses (Drosten *et al.*, 2003) and human enteroviruses 93 and 94, associated with acute flaccid paralysis (Junttila *et al.*, 2007). On the other hand, routine cell culture techniques available in most laboratories will not detect a number of clinically important viruses such as gastroenteritis viruses, hepatitis viruses, Epstein–Barr virus (EBV), human herpesvirus 6, 7 and 8 (HHV-6, -7, -8) and HIV. Other than HSV and some enteroviruses, most isolates of which will grow in human fibroblast cells within three days, the time taken for CPE (or, for example, haemadsorption) to develop for most clinical viral isolates is between 5 and 21 days, which is often too long to influence clinical management. For this reason, a number of modifications to conventional cell culture have been developed to yield more rapid results. These include centrifugation of specimens on to cell monolayers, often on cover slips, and immunostaining with viral protein-specific antibodies at 48–72 hours post inoculation (shell vial assay) (e.g. Stirk and Griffiths, 1988). Such techniques can also be undertaken in microtitre plates (O'Neill *et al.*, 1996). Certain changes, for example in haemadsorption or pH, may precede the CPE and therefore can be used to expedite detection of virus. Similarly, PCR techniques (see later) can be used to detect virus in cell culture supernatants before the appearance of CPE.

The role of conventional cell culture for routine diagnosis of viral infections has been a subject of active debate within the virology community (Carman, 2001; Ogilvie, 2001). Many laboratories are discontinuing or downgrading virus isolation methods in favour of antigen or genome detection for the rapid diagnosis of key viral infections, for example respiratory and herpes viruses. Nevertheless, it is important for certain reference and specialist laboratories to maintain the ability to employ this methodology to obtain live virus isolates and allow unexpected and emergent viruses to be grown and recognized.

SEROLOGY

This term is often used to refer to diagnostic tests for the detection of specific antibodies. More properly, the term encompasses any testing of blood serum samples for the presence of a specific antigen or antibody. However, as both antigen and antibody assays are often applied to whole blood or plasma, or indeed to body fluids other than blood (e.g. cerebrospinal fluid (CSF), oral crevicular fluid), it is helpful to use the term to span all such testing. As will be seen, most of the techniques used for viral antigen detection can also be used for detection of specific antibody, and vice versa.

Antigen Detection

Immunofluorescence

IF is one of the most effective rapid diagnostic tests. Direct IF involves the use of indicator-labelled virus-specific antibody to visualize cell-associated viral antigens in clinical specimens. The indirect method utilizes a combination of virus-specific antibody (of a nonhuman species) and labelled anti-species antibody. Usually, the label used is fluorescein. The indirect method is more sensitive, since more label can be bound to an infected cell. Results can be available within 1–2 hours of specimen receipt. The success of the technique depends on adequate collection of

Table 1.2 Virus isolation

Advantages	Disadvantages
'Catch-all' (as long as viable within the chosen cell line(s))	Only detects 'viable' virus
Sensitive	Slow (conventional cell culture)
Generates isolate for further study, for example phenotyping	Multiple cell lines required
Can be adapted for a more rapid result	Labour intensive and requires skilled staff
	Safety concerns, laboratory security

Table 1.3 Antigen detection by immunofluorescence

Advantages	Disadvantages
Rapid (same day)	Labour intensive and requires skilled staff
Sensitive for some viruses (e.g. RSV)	Dependent on high-quality specimen Interpretation is subjective

cells in the particular sample to be examined, for example epithelial cells for respiratory virus antigens or peripheral blood mononuclear cells (PBMCs) for CMV. An advantage of IF is that microscopic examination of the fixed cells can determine the presence of adequate cell numbers for analysis (Table 1.3). Whenever it is employed, however, a trained microscopist is required to interpret results, which remain subjective.

The most common use of this technique is for the diagnosis of respiratory viral infections. A panel of reagents can be used to detect RSV, parainfluenza viruses, influenza A and B, metapneumovirus and adenovirus in multiple wells of a microscope slide. Compared to cell culture this technique is rapid and sensitive, especially for detection of RSV. The ideal specimen is a nasopharyngeal aspirate or a well-taken throat/nasal swab, most usually obtained from infants with suspected bronchiolitis, in whom a rapid result is invaluable for correct clinical management and implementation of infection control measures. There is increasing evidence that community- or nosocomial-acquired respiratory viruses lead to severe disease in immunocompromised patients (reviewed in Ison and Hayden, 2002), and it is important that bronchoalveolar lavage specimens from such patients with respiratory disease are also tested for these viruses, in addition to bacterial and fungal pathogens.

IF has been used widely for the direct detection of HSV and VZV in vesicle fluid, and has advantages over EM in both sensitivity and specificity. Detection and semi-quantification of CMV antigen-containing cells in blood can also be undertaken by direct IF (CMV/pp65 antigenaemia assay). This technique involves separating PBMCs and fixing them on a slide, followed by staining with a labelled monoclonal antibody directed against the matrix protein pp65. The frequency of positive cells can predict CMV disease in the immunocompromised patient (van der Bij *et al.*, 1989) and has been used quite extensively, though it is labour intensive. It needs large numbers of PBMCs, making it unsuitable for some patients. In addition, it requires a rapid processing of blood specimens if a reduction in sensitivity of detection is to be avoided (Boeckh *et al.*, 1994). PCR has therefore

become the method of choice for qualitative and quantitative detection of CMV as well as several other viruses.

Enzyme-linked Immunoassay (EIA), Chemiluminescent and Fluorescence-based Immunoassay

Solid-phase systems for antigen detection are still used widely. EIA is based on the capture of antigen in a clinical specimen to a solid phase (such as the base and walls of a well in a microtitre plate, or multiple magnetic microparticles/beads) via a capture antibody, and subsequent detection uses an enzyme-linked specific antibody that produces a colour change in the presence of a suitable substrate. EIA is also readily used for the detection of specific antibody as well as antigen (see later).

Elaboration of capture and detector antibody species has increased the sensitivity of EIA antigen-detection assays, which are widely used for hepatitis B virus (HBV) surface antigen (HBsAg) and 'e' antigen (HBeAg) detection and for HIV p24 antigen detection. Neutralization of the antigen reactivity by the appropriate immune serum can be used to confirm the specificity of the antigen reactivity. In primary HIV infection, HIV p24 antigen is present in the blood prior to the development of antibodies. Therefore, assays which detect this antigen in addition to anti-HIV antibodies reduce the diagnostic 'window period', that is the time from acquisition of infection to its first becoming detectable (Hashida *et al.*, 1996). Similar assays that detect hepatitis C core antigen in addition to anti-HCV (hepatitis C virus) antibodies have been proposed for testing donated blood.

Molecules with chemiluminescent properties, for example acridinium ester, which produces chemiluminescence in the presence of hydrogen peroxide, can be conjugated to antibodies/antigens and used instead of enzymes for immunoassay detection. Fluorescent labels are another alternative. The fluorescence emissions of chelates of certain rare earth metals—lanthanides, for example Europium—are relatively long-lived. Thus, the presence of an antigen or antibody labelled with a lanthanide chelate can be detected by measuring fluorescence intensity at a delayed time point after excitation, background fluorescence having completely died away. This is the principle of the time-resolved fluorescence assay (TRFA). Both chemiluminescent and TRF methods are very sensitive and highly amenable to automation in commercial systems.

Particle Agglutination Assays

Small latex particles coated with specific antibody will agglutinate in the presence of antigen, and their clumping together can then be observed by the naked eye. This

rapid assay can be used for rotavirus diagnosis, with an equivalent sensitivity to EM.

Immunochromatography

Several immunochromatography or 'dipstick' tests have been developed commercially as point of care tests (POCTs) that can be used either in the laboratory, in the clinic or at the patient's bedside for specific antigen detection. Examples include tests for influenza A and for rotavirus. Such dipstick tests require no special expertise and are quick to perform.

A detector reagent (virus-specific antibody conjugated to a coloured indicator) is impregnated at one end of a base membrane in a disposable 'dipstick'. A capture antibody is coated on the membrane at the test region. When the clinical specimen is added to the sample pad, any viral antigen present in the specimen binds to the detector reagent and is carried along the membrane by capillary action. As the specimen passes over the test region coated with capture antibody, the viral antigen-bound detector reagent is immobilized. A coloured band proportional to the amount of virus present in the sample develops. The excess unbound detector reagent moves further up the membrane and is immobilized at the control band by an anti-detector antibody, and a second coloured line appears. Thus, two coloured lines on the test stick indicate the presence of virus. In the absence of virus in the patient's sample only the control band appears.

Antibody Detection

Viral infections generate a host immune response, and this can be used for diagnostic purposes. The classical response pattern following an acute infection is illustrated in Figure 1.1. The functional nature of this response is extremely variable. In some instances the antibodies are neutralizing and can be assessed for this activity (e.g. polioviruses). Other infections are controlled more effectively by T-cell responses, though the detection of antibody may still be used diagnostically.

Traditional methods of antibody detection did not distinguish between IgG and IgM responses, and diagnosis was simply based on seroconversion or a significant rise in antibody titre between acute and convalescent samples (10–14 days apart). The complement fixation test (CFT) was widely used in this way, though assay insensitivity and the cross-reactivity of many antigens used within the assay limited its clinical usefulness and, importantly, the diagnosis could only be made a week or more after the acute illness. The principle of complement fixation is that a specific reaction between an antigen and an antibody takes up complement. If a measured amount of complement is added to a reaction

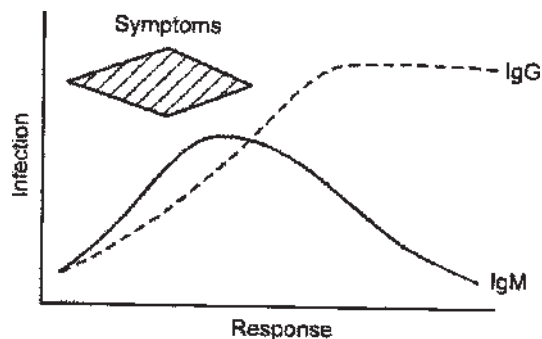


Figure 1.1 Typical evolution of antibody responses following an acute viral infection.

in which both antigen and specific antibody are present, the uptake of complement can be detected by a second 'detector' reaction in which sensitized red cells are added to the system. Failure to lyse the red cells signals that complement fixing antibody has been detected, giving a positive test result. Currently, complement-fixation assays are mostly used for the retrospective diagnosis of 'atypical' pneumonia (*Chlamydia psittaci/pneumoniae*, coxiella, influenza or mycoplasma).

Serum or plasma is the specimen of choice for antibody detection, but oral crevicular fluid (obtained by rubbing an absorbent sponge around the gums) can be used as a non-invasive alternative. This may be useful for outreach surveillance studies (Hope *et al.*, 2001; MMWR, 2007) or in children (Holm-Hansen *et al.*, 2007). In patients with viral central nervous system (CNS) infections, the CSF may be tested for virus antibodies, and the antibody concentration compared with serum to confirm intrathecal antibody synthesis.

Enzyme-linked Immunoassay (EIA) for Antibody Detection

Solid-phase enzyme immunoassays (EIAs), in which one of the reagents is immobilized on a plastic or other surface (e.g. magnetic beads), are used extensively in diagnostic laboratories. The use of synthetic peptides or recombinant antigens instead of whole viral lysates, and improvements in signal detection, have led to more sensitive, specific and rapid methods for measuring virus-specific antibody levels. The immunoassay format is versatile, and new assays can be designed quickly to cope with clinical demands, for example the investigation of new viruses such as SARS-associated coronaviruses. As indicated above, several non-isotopic, non-enzymatic reagent labelling and detection methods have been developed, such as chemiluminescence- and fluorescence-based methods. These are very sensitive, very rapid and highly amenable to incorporation into commercial automated systems.

Solid-phase immunoassays for the detection of antibody are essentially of three types (Figure 1.2):

- *Indirect assays.* Viral antigen is immobilized on to a solid phase. Specific antibody in the patient serum sample binds to this antigen and, after a washing step, is detected by an enzyme-labelled anti-human immunoglobulin. In this way, either specific IgG or IgM can be detected, depending on the indicator immunoglobulin (Figure 1.2a,b). Detection of IgM species is dependent on the prevailing level of IgG, and a high level of specific IgG may reduce the sensitivity of an IgM assay for the same virus. If rheumatoid factor is present in the clinical sample it may lead to false-positive IgM reactions (Figure 1.2c).
- *Capture assays.* IgG or IgM species are captured on to the solid phase by anti-human immunoglobulin, after which antigen and then labelled antibody is added. This is the preferred method for IgM assays, as it reduces the potential for interference by rheumatoid factor (Figure 1.2d).
- *Competitive assays.* In this case, a labelled antibody in the EIA system competes for binding to immobilized antigen with antibody in the clinical sample. This assay may improve the specificity of the assay diagnosis (Figure 1.2e).

Other Antibody Detection Methods

Immunoblot (western blot) methods can be useful for confirmation of certain infections, such as human T lymphotropic virus (HTLV) and HCV infection. These are based on the detection by antibodies within a serum sample of multiple antigenic epitopes previously separated and blotted onto a membrane. Nonspecific reactions within EIAs can be clarified in these systems, since the nonspecific antibodies will react with the nonviral antigenic epitopes, and the specific ones with the viral epitopes. Immunoblot assays are expensive and technically demanding.

Other antibody-detection techniques include haemagglutination inhibition, latex agglutination (in which antibody is captured by antigen-coated particles) and IF (most widely used for EBV diagnosis), and these techniques still have a significant role in clinical laboratories.

The diagnosis of acute infection by detection of specific antibody in body fluids is particularly suited to situations in which detection of the virus itself is difficult and time-consuming, or where virus excretion is likely to have ceased by the time of investigation, such as hepatitis A, rubella and parvovirus B19. There are situations, however, where IgM is produced over a prolonged period, or in response to re-infection, as is the

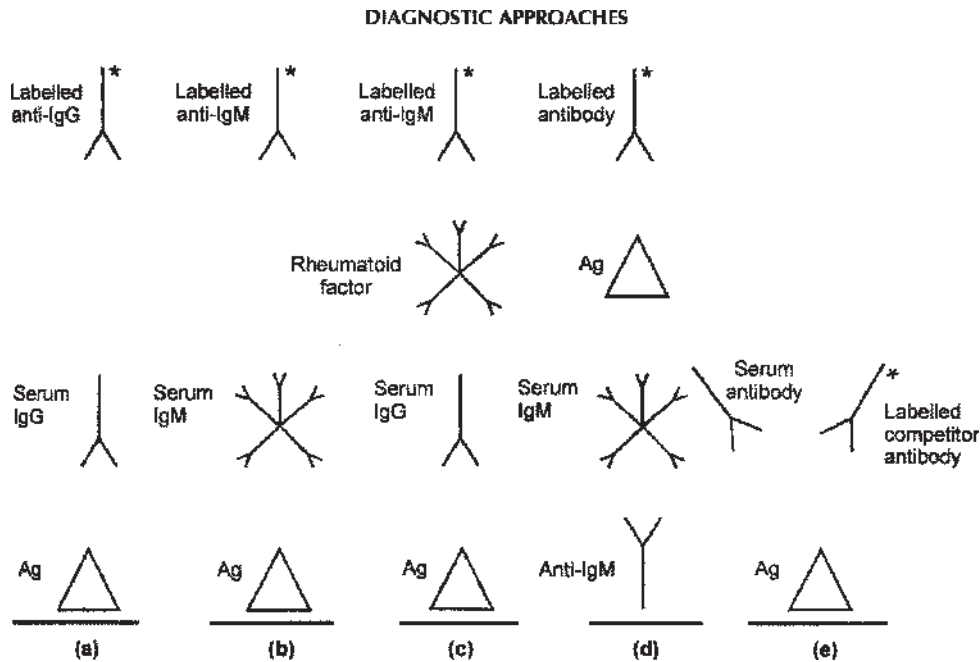


Figure 1.2 EIA formats (a) indirect IgG assay; (b) indirect IgM assay; (c) rheumatoid factor interference in IgM assay (indirect); (d) IgM capture assay; (e) competitive assay. Note that the solid horizontal lines represent the solid phase.

case for CMV. In these cases, past infection can better be distinguished from recent infection by antibody avidity tests. These are based on the principle that antibody responses mature over time, with high-avidity antibodies predominating at the later stage. By using a chaotropic agent (e.g. urea) during the EIA washing stage, low-affinity antibodies (representing recent infection) will be preferentially dissociated from antigen compared to higher-affinity antibodies (Blackburn *et al.*, 1991).

Antibody detection is also essential for diagnosis of, and screening for, persistent infections where antibodies are detectable in the presence of virus replication, such as HIV and HCV. The availability of sensitive and specific assays allows widespread screening for immunity against, for example, HBV, rubella, VZV and hepatitis A.

Despite recent advances in antibody-detection techniques, there remain inherent limitations to this form of virological diagnosis (Table 1.4). It is highly dependent on the ability of the individual to mount appropriate immune responses to infection. Thus, these methods have a limited role for diagnosing viral infections in severely immunocompromised patients (Paya *et al.*, 1989). Every effort must then be made to detect the virus itself. Transfusion or other receipt of blood products may lead to spurious serological results; for instance, leading to a false interpretation that a seroconversion, indicating acute infection, has occurred. The major role of antibody-detection tests in transplant patients is in identifying immune status at the baseline in order to forecast the risk of primary infection, re-infection or reactivation during subsequent immunosuppression (see later).

Interpretation of Serological Assays

Viral serology should take account of the patient's history and symptoms, and sometimes additional information may need to be sought. For example, the detection of very low levels of HBsAg in the absence of any other markers of hepatitis B infection might be explained by very recent immunization (because the vaccine itself is recombinant HBsAg) rather than hyperacute infection with this virus. The diagnosis of a primary virus infection can be made by demonstrating seroconversion from a negative to a positive specific IgG antibody response, or by detecting virus-specific IgM. A fourfold rise in IgG antibody titre between acute and convalescent samples can also be indicative of a primary infection (e.g. by CFT). Detection of virus-specific IgG without IgM in a single sample, or no change in virus antibody titre between acute and convalescent phase sera, indicates exposure to the virus at some time in the past. Results of antibody-detection assays can be complicated by a number of factors: the age of the patient (the production of serum IgG or IgM antibodies can be absent or impaired in the immunocompromised, neonates and the elderly), receipt of blood products with passive antibody transfer, maternal transfer of IgG antibodies and nonspecific elevation of certain virus antibodies due to recent infection with other viruses. The last phenomenon is particularly common with herpes virus infections, which have group-specific cross-reacting epitopes. IgM antibodies may persist for extended periods of time following primary infection, and may also be produced as a result of reactivation of latent infection, although not reliably so (e.g. CMV, EBV). The production of

Table 1.4 Serology

Advantages	Disadvantages
Specific IgG assays good indicator of prior infection	Retrospective (e.g. rising CFT titres). CFTs insensitive, especially to assess previous infection
Capture IgM assays good indicator of recent infection	Cross-reactivity and interference
May allow retrospective diagnosis if no acute specimen obtained	Poor sensitivity for diagnosing some congenital infections, for example CMV
Readily automated	Not appropriate for the immunocompromised
Rapid (same day)	Spurious results possible following receipt of blood products
Diagnosis of unculturable or poorly culturable viruses, for example hepatitis B	
Can use non-invasive samples such as saliva or urine	

CFT = complement fixation test.

virus-specific intrathecal antibody (requiring demonstration of an intact blood–brain barrier) can confirm the diagnosis of viral CNS infection, for example subacute sclerosing panencephalitis. Antibody-detection assays may be complemented and confirmed by molecular assays, for example PCR for HCV RNA in the presence of hepatitis C antibody or PCR for HIV provirus, used for the investigation of infants born to HIV-infected mothers.

MOLECULAR AMPLIFICATION TECHNIQUES

This is the most rapidly developing area in diagnostic virology, providing both qualitative and quantitative results. PCR and other molecular amplification techniques have now been applied to the diagnosis of virtually all human viruses and, in general, the sensitivity of these assays far exceeds that of other virus detection systems. However, the interpretation of results in a clinical setting may be difficult. A number of commercial kits and automated systems are now available, with the advantages of improved quality control and reduced inter-laboratory variability. The advantages and disadvantages of molecular techniques are summarized in Table 1.5. These issues will be discussed following a brief review of the techniques available.

The Principle of the Polymerase Chain Reaction (PCR)

This technique uses a thermostable DNA polymerase to extend oligonucleotide primers complementary to the viral DNA genome target (Saiki *et al.*, 1988). Consecutive cycles of denaturation, annealing and extension result in an exponential accumulation of target DNA. This is

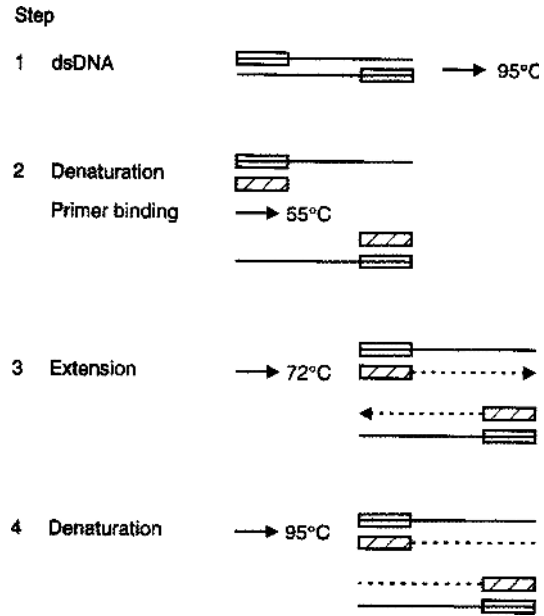


Figure 1.3 Polymerase chain reaction.

limited only by substrate (nucleotide) availability and possible competition between target genome and nontarget amplicons for reaction components (Figure 1.3). RNA genomes require transcription to complementary DNA (reverse transcription) prior to the PCR reaction. Undertaking a second round of PCR on the first-round amplicon can increase the overall sensitivity of detection (nested PCR). The second round uses a different set of PCR primers internal to the first set, and can therefore act as confirmation that the correct amplicon was produced by the first-round reaction.

Table 1.5 Molecular assays

Advantages	Disadvantages
Speed—results available in a few hours	Commercial assays expensive (but becoming relatively less so)
High sensitivity—gold standard for many viruses	High set-up costs (equipment) for ‘in-house’ assays
Wide range of applications/versatility	Susceptible to contamination
Increasing availability of automation	Rigorous quality-control systems required for ‘in-house’ molecular diagnostics
Increasing availability of commercial assay kits with built-in quality control	Some assays lack clinical validation
Increasing availability of external quality-assurance programmes	Lack of availability and expertise outside specialist centres
Amplicon can be used for sequencing/genotyping	No isolate available for phenotyping
Can be highly specific to viral subtype	Target sequence must be known and highly conserved
Quantification readily possible	
Detection of uncultivable viruses (e.g. acute HCV infection)	
Can use non-invasive samples, for example urine, saliva	

Primers

The correct choice of primers is an important determinant of the success of any PCR. The nucleic acid sequence of at least a part of the viral genome needs to be known, and primers must target a very well-conserved region. This can be done using multiple alignment programs; however, the final success of the PCR depends on the availability of sequence data from a range of different viral isolates. Otherwise, unusual viral variants may not be detected. This issue is as important for commercial assays as it is for 'in-house' assays, as was demonstrated by suboptimal HIV subtype detection by a commercial quantitative PCR (qPCR) assay (Arnold *et al.*, 1995). Other important aspects of primer design include the avoidance of secondary structure or complementarity between primers (leading to so-called primer-dimer amplification artefacts). Computer programs used to design primer sequences address these problems.

Preparation of Clinical Specimens for PCR

Viral gene detection methods do not rely on persistence of viral infectivity within the clinical specimen and in one respect this is a major advantage over traditional methods of virus detection. Specimens should be transported and stored in the refrigerator or freezer prior to analysis, but less meticulousness is required than to achieve virus isolation. However, viral RNAs are susceptible to nucleases, present in all biological material, and certain specimen types (e.g. intraocular fluids (Wiedbrauk *et al.*, 1995) and urine (Chernesky *et al.*, 1997)) contain inhibitors of PCR. PCR is therefore susceptible to false-negative results, and specimens for qualitative and especially qPCR require careful preparation. Each assay needs to be evaluated for individual specimen type and patient group. For blood samples, the anticoagulant heparin is contraindicated because it inhibits the PCR reaction. It is generally recommended that for viral quantification ethylenediamine tetraacetic acid (EDTA) anticoagulated blood is separated as soon as possible, after which the plasma can be stored frozen until analysis. If multiple tests are to be undertaken on one sample, it should be aliquoted on receipt to avoid multiple freeze-thawing. A number of different nucleic acid extraction methods are available. The choice depends on the nature of the clinical specimen and whether the target is RNA or DNA.

Detection of Conventional PCR Product

The PCR product of any specific reaction has a known size, and can therefore be detected on an agarose gel, usually by staining with ethidium bromide (or other, potentially safer, fluorescent stains such as SYBR Green) in comparison with a molecular weight ladder. However,

more than one specific band may be seen, or the band may not be of the expected size. For this reason, detection of the product by hybridization with a specific nucleic acid probe is desirable. A microtitre plate format with a colorimetric end point read by a standard spectrophotometer may be used, for example (Gor *et al.*, 1996). Many commercial PCR assays employ this system. The addition of such a step enhances the specificity of the assay, and may improve sensitivity.

Multiplex PCR

Since more than one viral target is frequently sought in each specimen, multiplex assays have been devised in which multiple sets of primers (against different targets) are combined within one PCR reaction (for example Dingle *et al.* 2004). Each set of primers requires specific conditions for optimal amplification of the relevant target, and the development of a multiplex system requires a detailed evaluation of these conditions to ensure that the efficiency of amplification for any one target is not compromised. Identification of the specific product in this system may be based on the different size of amplicons, or the use of different probes. Figure 1.4 illustrates a multiplex PCR for RSV A (RA), RSV B (RB) and human metapneumovirus with agarose gel-based detection. Increasingly, conventional multiplex PCR has been replaced by multiplex real-time PCR (see below).

Quantification

Conventional PCR is inherently a qualitative assay. Initial attempts to quantify with PCR involved the simultaneous analysis of samples with a known target genome copy number and comparing the intensity of bands on an agarose gel with that of the test specimen. However, the efficiency of amplification within any one PCR reaction can be exquisitely sensitive to changes in reaction conditions and inhibitory factors present in the clinical specimen. It is therefore important that internal standards (within the same PCR reaction) are used for quantitative competitive (qcPCR) assays. These control sequences should mimic the target genome as closely as possible, yet be detectable as a distinct entity on final analysis. This can involve the incorporation of restriction enzyme sites at which the control amplicon, but not the target sequence, can subsequently be cleaved (Fox *et al.*, 1992), or involve use of a control sequence of different size (Piatak *et al.*, 1993). Commercial assays often use a jumbled sequence as a control, with subsequent use of probes against both control and target sequences. In all cases, since the number of input control genomes is known, simple proportions can be applied to the signals to generate a quantitative value for the clinical specimen (Figure 1.5a). Real-time

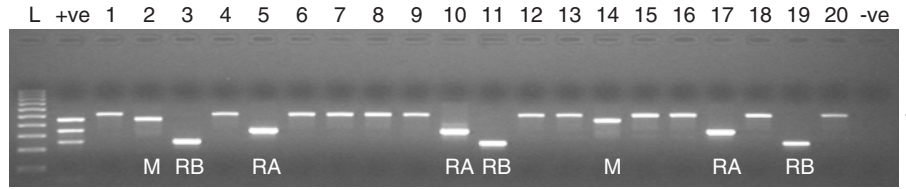


Figure 1.4 A 2% agarose gel of ethidium bromide-stained products from an internally-controlled nested multiplexed reverse transcriptase PCR for RSV A (RA), RSV B (RB) and human metapneumovirus (M). Lanes 1–20 represent 20 clinical samples; L size markers; +ve positive control; –ve negative control. Each clinical sample has been spiked with an internal control from the hepatitis delta genome. The internal control reaction is out-competed by the amplification of target genome in this assay. The asterisk indicates the position of the internal control when target viruses were not detected. (Source: Based on a figure in Dingle *et al.*, 2004.)

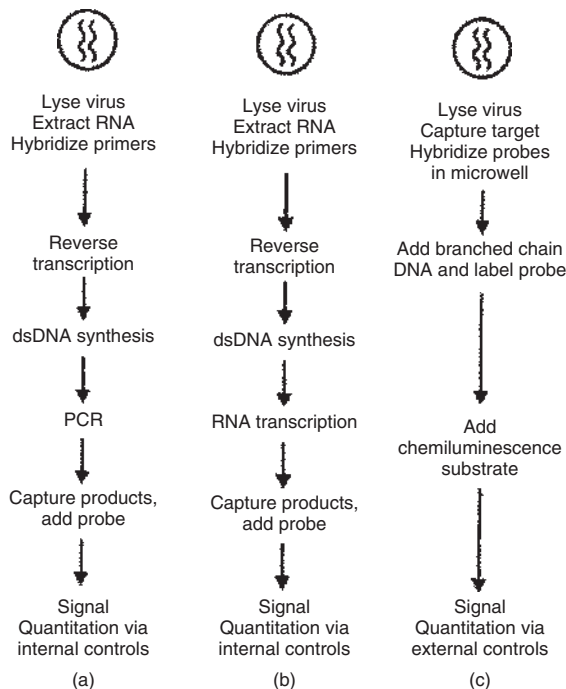


Figure 1.5 Quantitative molecular methods for HIV plasma RNA. (a) PCR; (b) NASBA; (c) bDNA.

PCR methods, as described in the next section, have now largely replaced these qcPCR assays.

Real-time PCR

The conventional PCR method described above aims to maximize the amplification reaction and depends on end-point detection of product. More sensitive detection methods allow the kinetics of the amplification to be measured, and may require fewer cycles of amplification for the product to be detected.

Real-time PCR systems allow the reactions to be undertaken within a closed system, and fluorescence generated

by the assay can be measured without further manipulation. Some of these systems produce very rapid temperature cycling times and, by also abandoning post-PCR detection procedures, this means that PCR tests can be completed within minutes. Many of the signalling technologies (reviewed in Mackay, 2004) rely on energy transfer between a donor fluorophore and a proximal acceptor molecule (fluorescence resonance energy transfer, FRET). The simplest of these involves the use of molecules, such as SYBR green, which spontaneously intercalate into dsDNA and then fluoresce when exposed to a suitable wavelength of light. Specificity of the PCR reaction for the correct product (rather than artefacts) is provided by analyzing a decrease in fluorescence at the melting (denaturation) temperature specific for that product.

$5'$ nuclease or TaqMan oligoprobes (see Figure 1.6a) utilize the intrinsic $5' \rightarrow 3'$ endonuclease activity of *Taq* DNA polymerase. A short target-specific probe, in which the fluorescence of the fluorophore at the $5'$ end is quenched by the fluorophore at the $3'$ end, binds to the relevant amplicon, and subsequent hydrolysis of this probe increases fluorescence (Morris *et al.*, 1996).

The method of choice for amplicon detection in the LightCycler system employs linear oligoprobes (or 'kissing' probes: see Figure 1.6c), one bearing a donor fluorophore and the other an acceptor fluorophore. Adjacent hybridization of the two probes on the denatured amplicon DNA results in a FRET signal due to interaction between the donor and acceptor.

Somewhat similarly to $5'$ nuclease probes, hairpin oligoprobes (see Figure 1.6c) carry a fluorophore and quencher at opposite ends. The labels are held in close proximity by homologous base-pairing of the distal ends of the oligonucleotide into a hairpin structure. Hybridization of the probe to the target separates fluorophore and quencher, resulting in increased fluorescence. The self-fluorescing amplicon concept is similar to that of the hairpin oligoprobe except that the fluorophore and quencher are attached to opposite ends of a primer (rather than a probe), distal complementary sequences of which

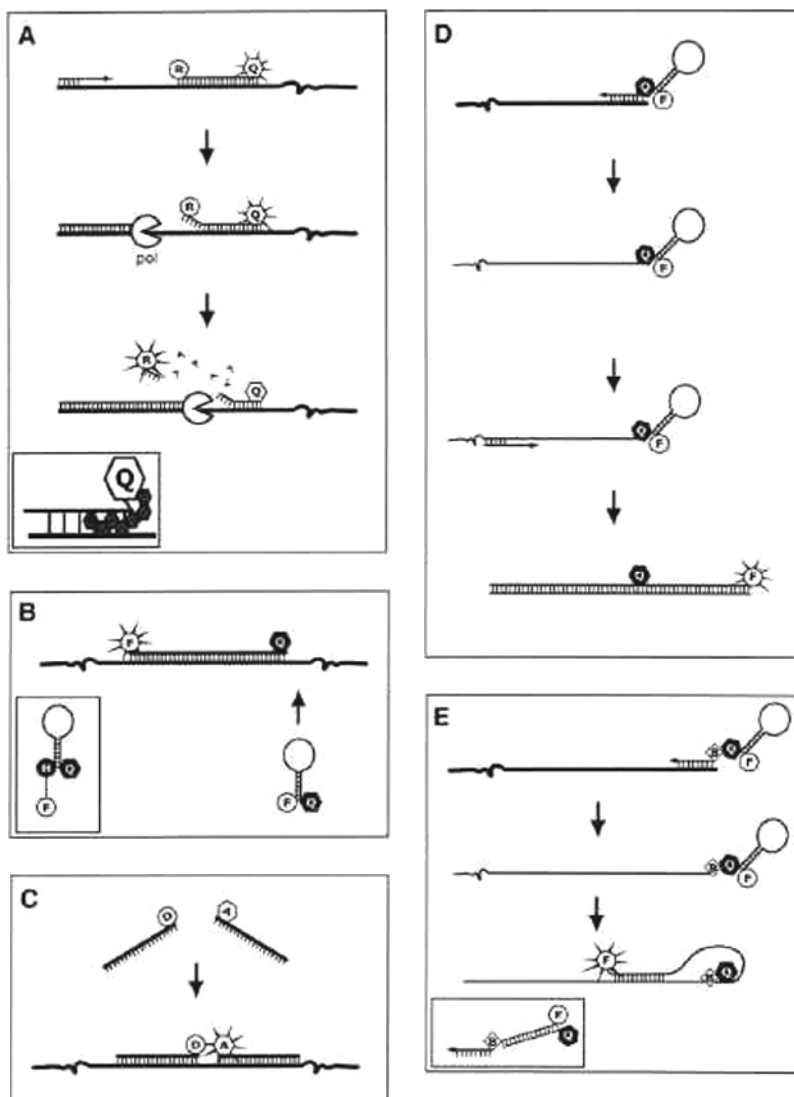


Figure 1.6 Oligoprobe chemistries. (a) 5' nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyzes the oligoprobe via its 5' → 3' endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q, open), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher. Inset shows the non-fluorescent quencher (NFQ) and minor groove binder (MGB) molecule that make up the improved MGB nuclease-oligoprobes. (b) Hairpin oligoprobes. Hybridization of the oligoprobe to the target separates the fluorophore (F) and nonfluorescent quencher (Q, closed) sufficiently to allow emission from the excited fluorophore, which is monitored. Inset shows a wavelength-shifting hairpin oligoprobe incorporating a harvester molecule. (c) Adjacent oligoprobes. Adjacent hybridization results in a FRET signal due to interaction between the donor (D) and acceptor (A) fluorophores. This bimolecular system acquires its data from the acceptor's emissions in an opposite manner to the function of nuclease oligoprobe chemistry. (d) Sunrise primers. The opposite strand is duplicated so that the primer's hairpin structure can be disrupted. This separates the labels, eliminating the quenching in a similar manner to the hairpin oligoprobe. (e) Scorpion primers. The primer does not require extension of the complementary strand; in fact it blocks extension to ensure that the hairpin in the probe is only disrupted by specific hybridization with a complementary sequence designed to occur downstream of its own, nascent strand. Inset shows a duplex scorpion that exchanges the stem-loop structure for a primer element terminally labelled with the fluorophore and a separate complementary oligonucleotide labelled with a quencher at the 5' terminus. (Source: Reproduced from Mackay *et al.*, 2002. *Nucleic Acids Research* 30(6) pp. 1292–1305 Figure 3 (A-E), with permission from Oxford University Press.

keep the fluorophore and quencher in close proximity (see Figure 1.6d). On hybridization of the primer to its target, the fluorophore and quencher are separated from one another, and irreversibly incorporated into the PCR product.

Due to the limited number of fluorophoric labels available and the significant overlap in their emission spectra, quantification of multiplex reaction products is difficult and often not possible for more than two or three targets. Moreover, one channel is required for the detection of an internal control in order to confirm satisfactory extraction and amplification (see 'Quality Control', later). Nevertheless, a recent study reported a 'pentaplex' assay using 5' nuclease probes for four viral targets (influenza A, influenza B, adenovirus and enterovirus) together with an internal control sequence (Molenkamp *et al.*, 2007). Development of novel chemistries and improvements in real-time instrumentation and software should allow more fluorophores to be multiplexed and enhance real-time PCR assays.

The major advantage of real-time PCR is that it is inherently semi-quantitative: the quantity of target sequence present in the initial reaction mixture determines the number of temperature cycles required for a threshold fluorescence signal to be reached. An external standard curve is used to determine the relationship between cycle threshold (Ct) and input target copy number. An example of real-time detection of a calibration series for the detection of hepatitis C is shown in Figure 1.7. The dynamic range of real-time PCR of at least eight \log_{10} copies of template surmounts the problem encountered by many qPCR reactions of inability to quantify high virus loads if sensitivity at the lower end of the assay is also to be maintained

(Garson *et al.*, 2005). In addition, intra- and inter-assay variability is reduced in comparison with qPCR (Abe *et al.*, 1999; Locatelli *et al.*, 2000).

The disadvantages of real-time PCR compared with conventional PCR include an inability to monitor the size of the amplicon or to perform a nested PCR reaction without opening the system; incompatibility of some systems with certain fluorescent chemistries; and (as discussed above) relatively limited capability for multiplexed reactions because of the few non-overlapping fluorophores available. In addition, the start-up costs of real-time PCR may be prohibitive. Despite these difficulties, real-time PCR is now used routinely in many diagnostic virology laboratories, for both qualitative and quantitative applications. As with conventional PCR, real-time PCR has proven cost-effective in high-throughput laboratories when compared with traditional culture-based methods of viral diagnosis.

PCR Contamination and Control Reactions

PCR is highly susceptible to contamination from amplified products generated in a previous reaction, from target sequences cloned in plasmid vectors and from other infected clinical specimens. By contrast, a false-negative result can arise from inadequate nucleic acid extraction from a sample, or from inhibitory factors in the PCR reaction; or the sensitivity of the assay, though reduced, may not be completely inhibited. Relevant controls within each PCR run are essential for a correct interpretation of a positive or negative result, and these are highlighted in Table 1.6. The limit of sensitivity for each assay must be assessed. This can be undertaken by serial dilutions

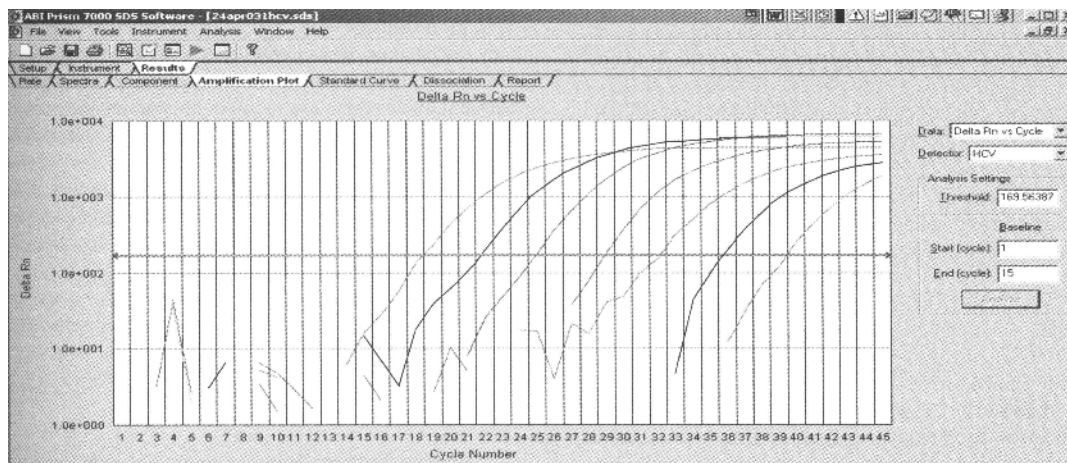


Figure 1.7 Real-time PCR detection of a hepatitis C calibration series from 10 million IU/ml down to 10 IU/ml in 1 log steps. x axis = cycle number; y axis = log fluorescence. The sample with the highest virus load (10^7 IU/ml) requires 39 cycles of PCR before reaching a detectable level. Results obtained by Dr Jeremy Garson.

Table 1.6 PCR—recommended controls

Negative controls	
Extraction control	to control for contamination during extraction (use negative clinical material)
Reagent control	to control for contamination of reagents (use solvent in which extracted nucleic acid is suspended)
Positive controls	
Extraction control	Use positive clinical material
Control genome/run control	To control for PCR efficiency, specifically to assess sensitivity
Alternate target/internal control	to control for inhibition of reaction

of a tissue culture supernatant of known median tissue culture infective dose (TCID₅₀) or virion concentration (as measured by EM), or of a preparation of purified viral genome provided at a standardized concentration. Alternatively, plasmid containing the target genome may be used, but many laboratories are reluctant to introduce plasmids into the molecular biology area because of the risk of widespread contamination with plasmid amplicons.

There are two specific procedures designed to reduce PCR contamination. Firstly, extraneous DNA contaminating PCR reagents can be inactivated by subjecting 'clean' PCR reagents to ultraviolet irradiation. This introduces thymidine dimers into the DNA chain, rendering it unamplifiable. More effective is the substitution of dUTP for dTTP in the PCR reaction (Longo *et al.*, 1990); this does not affect specific product detection. The use of uracil DNA glycosylase in any subsequent PCR reaction prevents DNA polymerization of any uracil-containing DNA, but has no effect on thymidine-containing DNA template. Thus any contaminating DNA from a previous reaction is not amplified.

Physical Organization of the Laboratory for PCR

The physical requirements for undertaking 'in-house' PCR reactions are demanding (Victor *et al.*, 1993). A 'clean room' is required for preparation and aliquoting of reagents. This must be protected from any possible contamination with viral nucleic acid. A separate area is also required for nucleic acid extraction, although this can be undertaken in a diagnostic area. A dedicated PCR room is required for setting up reactions and siting thermal cyclers. Finally, another room is required for any post-PCR analyses, such as running gels. Dedicated laboratory coats and equipment are required for each of these areas, and strict adherence to protocol by all staff is essential.

The provision of such a dedicated set of rooms for molecular biology is a challenge for busy, crowded, diagnostic virology laboratories. Nevertheless, it is paramount that diagnostic PCR reactions are undertaken with the

risk of contamination minimized, and every effort must be made to provide the relevant space if such assays are to enter the routine diagnostic armamentarium. Some of the newer automated commercial assays incorporate several of the above steps within a self-contained machine. However, it is unwise to use such assays outside of a laboratory environment in which staff are well trained in this type of work.

The Range of Other Amplification Systems

Other, mostly commercial, amplification systems include the ligase chain reaction (LCR), which, as with PCR, requires a thermal cycler. Nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), strand displacement amplification (SDA) and branched chain DNA (bDNA) do not require any specialized thermal cycler. A number of newer technologies particularly suited to the simultaneous detection of multiple viral (and nonviral) targets in individual samples are also coming into use.

Ligase Chain Reaction

LCR involves hybridization of two oligonucleotide probes at adjacent positions on a strand of target DNA, which are joined subsequently by a thermostable ligase. The reaction also takes place on the complementary strand so multiple rounds of denaturation, annealing and ligation lead to an exponential amplification of the viral DNA target (Hsuih *et al.*, 1996). RNA targets require prior reverse transcription.

Nucleic Acid Sequence-based Amplification

This technique uses RNA as a target, utilizing three enzyme activities simultaneously: reverse transcriptase (RT), RNase H and a DNA-dependent RNA polymerase (Guatelli *et al.*, 1990). A DNA primer incorporating the T7 promoter hybridizes to the target RNA and is extended by RT. RNase degrades the RNA strand, and the RT then

utilizes a second primer to produce double-stranded DNA. Subsequently, T7 polymerase forms multiple copies of RNA from this DNA template. This method is suited to the detection of RNA viruses, or mRNA transcripts of DNA viruses. In addition, it can be turned into a quantitative assay using internal controls (Figure 1.5b). Different detection formats for the amplified RNA product have been developed, including electrochemiluminescence and molecular beacon detection technologies, and adapted for rapid detection of various viruses, for example West Nile and St Louis encephalitis (Lanciotti and Kerst, 2001). TMA techniques are very similar. Commercial TMA systems have been used to detect and quantify HIV and HCV nucleic acid sequences.

Strand Displacement Amplification

In this technology, an oligonucleotide primer containing a restriction enzyme site binds to its complementary (target) nucleic acid. An exonuclease-deficient DNA polymerase is used in the presence of dGTP, dATP, dUTP and a dCTP containing an α -thiol group (dCTP α S) to produce double-stranded DNA containing a restriction enzyme site. Upon binding, the restriction enzyme nicks the strand without cutting the complementary thiolated strand. The exo-DNA polymerase recognizes the nick and extends the strand from the site, displacing the previously-created strand. The recognition site is repeatedly nicked and restored by the restriction enzyme and exo-DNA polymerase, with continuous displacement of DNA strands containing the target segment. The process becomes exponential with the addition of an antisense primer containing the appropriate recognition site. SDA technology has been established in a fully-automated system known as BDProbeTec.

Hybridization Methods

These methods are based on the hybridization of a labelled oligonucleotide probe to a unique complementary piece of viral genome, and can be undertaken either on a solid phase or *in situ*. The short probes are 20–30 bases in length and can be RNA (riboprobe) or DNA. The bDNA assay is a modification of the probe assay principle and, unlike the other molecular methods described so far, it uses a *signal* amplification system rather than amplifying a *target* genome. A single-stranded genome (RNA or DNA) is hybridized to an assortment of hybrid probes, which in turn are captured on to a solid phase by further complementary sequences. Branched DNA amplifier molecules then mediate signal amplification via enzyme-labelled probes with a chemiluminescent output. This method can also provide quantitative results (De-war *et al.*, 1994; van Gemen *et al.*, 1993) (Figure 1.5c).

The hybrid-capture assay is another hybridization-based signal-amplification system in which riboprobes hybridize with DNA targets. These RNA-DNA hybrids are captured and detected by means of a labelled monoclonal antibody, which has been developed commercially and used extensively for the detection of HPV genome in cervical brushings/washings.

New Molecular Techniques

Loop-mediated Isothermal Amplification Assay

The loop-mediated isothermal amplification assay (LAMP) is another method for rapid amplification of DNA under isothermal conditions. It is based on the principle of autocycling strand-displacement DNA synthesis (Notomi *et al.*, 2000). The enzyme required is a DNA polymerase with high strand-displacement activity. A high degree of target specificity is achieved by the use of two outer primers and two inner primers, with each of the inner primers recognizing independent target sequences. The LAMP reaction results in the production of a mixture of stem-loop DNAs of different stem lengths, and cauliflower-like structures comprising multiple loops. These products can be detected by gel electrophoresis and appropriate staining, or (because pyrophosphate ion is a by-product of DNA synthesis) by monitoring the accumulation of precipitated magnesium pyrophosphate in a simple turbidometer (Mori *et al.*, 2004). Further assay refinements include the employment of an initial RT step, so as to be able to apply the technique to an RNA target, and the use of an additional pair of 'loop primers' to accelerate the LAMP reaction (Nagamine *et al.*, 2002). Very sensitive, specific and fast LAMP assays have been reported for detection of West Nile virus (Parida *et al.*, (2004)) and noroviruses (Yoda *et al.*, 2007). As the technique does not require sophisticated equipment, it is potentially valuable for use in resource-poor countries.

Microarrays

Microarrays consist of arrays of single-stranded DNA oligonucleotide probes spotted on to specific locations on a small glass slide, membrane or coated quartz microchip surface. Tens of thousands of spots can be contained on one microarray. DNA and RNA is extracted from experimental or clinical samples, and then target sequences are amplified and labelled with fluorescent markers. When these labelled nucleic acids are hybridized to complementary sequences in the array, the amount of label can be monitored at each spot, thereby indicating whether or not the complementary nucleic acid sequence was present in the original sample. Microarrays, for example the Pneu-moVir from GENOMICA SA are now entering routine

laboratory use for the diagnosis of multiple viral respiratory tract pathogens at reasonable economic cost compared with multiple multiplexed conventional PCR-based assays. In the future it is likely that they will be developed for the simultaneous detection of multiple infectious agents—bacterial, viral and fungal—in individual patient samples. By detecting specific target sequences, not only should they be able to detect the presence of a specific pathogen but they should also determine whether that pathogen demonstrates genotypic drug resistance.

Micro-bead Suspension Array Multiplex PCR

This technology combines multiplex PCR for simultaneous amplification of multiple target sequences with a coloured micro-bead detection system. For each pathogen, target-specific capture probes are covalently linked to a specific set of colour-coded beads. Labelled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A dual-laser detection device identifies the colour of each bead (corresponding to a particular pathogen) and determines whether labelled PCR product is present on the bead or not, so indicating the presence or absence of the particular pathogen in the original sample. The technology offers the potential for the rapid simultaneous detection and quantification of up to 100 different analytes within a single sample. Recent studies have applied it successfully to respiratory virus diagnosis (Mahony *et al.*, 2007) and to determination of HPV type (Schmitt *et al.*, 2006).

Quality Control for Molecular Methods

Compared to more traditional virological methods, molecular biological ones are expensive. There have recently been initiatives to make all molecular assays in Europe compliant with the *in vitro* diagnostic directive (IVDD). However, many laboratories in the United Kingdom and elsewhere continue to use well-validated molecular assays developed in-house. This makes it difficult for each laboratory to evaluate each molecular assay, and there is likely to be considerable inter-assay and inter-laboratory variation (Valentine-Thon, 2002). If a noncommercial assay is employed, the critical reagents should be batch tested, and it is vital that a known low-level positive 'run control' is used to monitor within- and between-analytical run variability. To supplement these internal quality control measures, it is very important to participate in external quality assessment (EQA), since major clinical and therapeutic decisions are made on the basis of molecular assay results. An EQA service sends participating clinical laboratories samples on a regular basis, which they test as if they had come from patients. Results are returned to the EQA centre, which provides a report that compares

a participant's performance with that of all laboratories and/or groups of laboratories using similar test methods. These may be commercial assays or developed 'in-house'. Programmes such as Quality Control for Molecular Diagnostics (www.qcmd.com) provide EQA schemes for blood-borne viruses and other pathogens such as CMV, enterovirus and respiratory viruses. Participation in such schemes represents a significant but essential expenditure for diagnostic laboratories.

Automation of Molecular Techniques

With the use of highly-sophisticated robotics, the component processes of molecular assays—extraction of nucleic acid, real-time PCR reaction set-up, amplification and detection—can also be automated. Thus, it is possible for a single machine to perform a specific diagnostic nucleic acid test on a patient sample and deliver a very rapid result without any technical expertise being required at all, for example the GeneExpert System. Such self-contained, fully-integrated systems are currently very expensive however, prohibiting widespread use.

Clinical Value of Molecular Techniques

The application of qualitative and quantitative molecular analysis to human viral infections has provided new insights into the natural history of infections such as HIV, HBV, HCV and the herpesviruses. This includes the nature of viral persistence and latency, viral replication and turnover rate, and an understanding of the response to antiviral therapies. Molecular diagnostic assays have not merely increased sensitivity over alternative methods; they have resulted in the identification of a number of new viruses associated with respiratory disease: coronaviruses NL63 (van der *et al.*, 2004) and HKU1 (Woo *et al.*, 2005), and human bocavirus (Allander *et al.*, 2005).

Diagnosis of Virus Infection and Disease

Infection is revealed by the detection of virus in a clinical specimen. The infection may be asymptomatic or symptomatic (disease). However, the key determinant for correct diagnosis is the sensitivity of the assay, with a goal of detecting viral genome if it is present. A sensitive qualitative assay is relevant, for instance, in the diagnosis of HIV in infants (proviral DNA in PBMCs) (Lyll *et al.*, 2001) or acute HCV (plasma/serum RNA) infection (Aarons *et al.*, 2004). Before introducing such an assay into routine use, the sensitivity and specificity of the new test must be established, according to the formulae in Table 1.7. Note that in this instance, these parameters are compared to an existing gold standard assay (true positives or negatives) and therefore relate purely

Table 1.7 Evaluation of a new diagnostic assay

Parameter	Description	Formula
Sensitivity	Proportion of true positives correctly identified by test	$\frac{\text{true positive results}}{(\text{true positives} + \text{false negatives})}$
Specificity	Proportion of true negatives correctly identified by test	$\frac{\text{true negative results}}{(\text{true negatives} + \text{false positives})}$
Positive predictive value	Proportion of patients with positive test results who are correctly diagnosed	$\frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$
Negative predictive value	Proportion of patients with negative test results who are correctly diagnosed	$\frac{\text{sensitivity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})}$
Likelihood ratio	Indicates how much a given diagnostic test result will raise or lower the pretest probability of the target disorder	$\frac{\text{sensitivity}}{(1 - \text{specificity})}$

to a comparison between assays. Since molecular assays are usually more sensitive than existing assays, it is often necessary to confirm by other means that the samples positive solely by the molecular assay are indeed true positives. This can be done by confirming the identity of the PCR product, or by correlation with another marker of infection (for instance, seropositivity, where appropriate) or with the clinical background. Thus, an expanded gold standard, including positives by both existing and new assay, is used for sensitivity and specificity calculations. A useful concept in evaluating a diagnostic test is the likelihood ratio (Table 1.7), which indicates how much a given diagnostic test result will raise or lower the pre-test probability of the target disorder (Altman and Bland, 1994).

As discussed above, the nature of viral disease has had to be redefined in the light of qualitative and quantitative molecular data. Increasingly, it is possible to detect the presence of virus at low genome copy number in the absence of symptoms. This may make the interpretation of positive results problematic, and requires close clinical-virological liaison. Two approaches are possible:

1. *Qualitative detection of viral genome at a site that is normally virus-free.* A good example is the diagnosis of viral encephalitis, in which detection of HSV, CMV, VZV or enterovirus genome is diagnostic (Jeffery *et al.*, 1997). Qualitative PCR offers significant advantages in terms of speed over traditional methods of viral diagnosis and, indeed, it has been very difficult traditionally to propagate herpesviruses in cell culture from CSF samples. It is unclear whether this is a reflection of a low level

of virus, or whether there is a preponderance of disrupted, non-infectious virus produced from brain tissue into the CSF. Early diagnosis and treatment of CNS infection can improve prognosis in herpes simplex encephalitis (Raschilas *et al.*, 2002), or can reduce unnecessary treatment and hospitalization as in the case of enteroviral meningitis (Nigrovic and Chiang, 2000).

2. *Qualitative detection of virus without an exquisite level of sensitivity.* This is useful where low-level viral shedding may occur in the absence of disease. An example is the use of PCR for HSV1 and HSV2 to determine the cause of oral/genital ulceration (do Nascimento *et al.*, 1998). Another example is the diagnosis of viral gastroenteritis by detection of rotavirus, norovirus or faecal adenovirus genome in stool samples (O'Neill *et al.*, 2002).

Staging of Infection and Prediction of Disease

For many persistent virus infections with transient or continual low-level viraemia, the onset of symptomatic disease is associated with a higher viral replication rate. This provides the rationale to identify the levels of viraemia that are predictive of disease. Quantitative molecular data on CMV disease in allogeneic bone-marrow transplant patients (Boeckh *et al.*, 1996) and AIDS patients (Spector *et al.*, 1998), and similar molecular data on BK polyomavirus-associated nephropathy in renal transplant recipients (Limaye *et al.*, 2001) demonstrate the usefulness of this approach. The

capacity of a positive laboratory test to predict disease must be established by detailed prospective surveillance protocols, in order to generate positive and negative predictive values (Table 1.7). Since the natural history of viral infections (relationship between replication and disease) may be influenced by factors such as the length and nature of immunosuppression, these parameters should be determined separately for different patient groups, such as human stem cell transplant (HSCT) recipients, solid organ recipients and patients with AIDS. Large prospective studies are therefore required in each case. Standardization within commercial assay systems and/or against international unitage standards will help in this respect.

qPCR for CMV has emerged as the preferred screening method for detection of CMV viremia in patients following allogeneic stem cell and solid organ transplant. Although there are currently no universally-accepted qPCR treatment thresholds at which to start pre-emptive therapy, evidence suggests that one of $\geq 10\,000$ copies/ml whole blood is a safe and effective strategy in clinically stable patients (Verkruyse *et al.*, 2006).

Data from the Multicentre AIDS Cohort Study (MACS) has shown that a high virus load predicts a faster rate of decline of CD4+ cells (Mellors and Rinaldo, 1996). This became a guideline for initiating antiviral therapy. However, more recently the British HIV Association (BHIVA) has moved away from recommending initiation of therapy based primarily on plasma HIV RNA load. It recommends that therapy for asymptomatic established infection should be deferred until the CD4+ cell count is between 200 and 350 cells/ μ l (Gazzard *et al.*, 2006). For hepatitis B infection, there is evidence that a high viral load predicts progression to cirrhosis (Iloeje *et al.*, 2006) and the development of hepatocellular carcinoma (Ohkubo *et al.*, 2002).

Genetic Subtyping (Genotyping)

HCV is a genetically heterogeneous virus with six major genotypes (Simmonds *et al.*, 1993). Some genotypes (namely types 2 and 3) have a more favourable response to interferon-based treatment than others (Chemello *et al.*, 1994; Hadziyannis *et al.*, 2004) and genotyping therefore affects the management of HCV infection. Sequencing is the reference method of HCV genotyping. Alternative methods include a line probe assay (in which biotinylated PCR product from the 5' untranslated region (UTR) is hybridized with subtype-specific oligonucleotide probes attached to a nitrocellulose strip and detected with a streptavidin-alkaline phosphatase conjugate), subtype-specific PCR and restriction fragment length polymorphism (RFLP).

Hepatitis B is a similarly heterogeneous virus. Several studies have shown that genotype B (prevalent in the Far

East) is associated with both a better overall prognosis (Kao *et al.*, 2002; Sakugawa *et al.*, 2002) and a higher rate of interferon-induced HBeAg clearance than hepatitis B genotype C (Wai *et al.*, 2002). HBV genotyping is likely to be used clinically in future, for example in guiding appropriate antiviral treatment. In HBeAg negative, antiHBe positive patients with discordantly high viral loads (2000 IU ml⁻¹), sequencing of HBV for the presence of pre-core and core promoter mutations is becoming a common request.

Over 70 genotypes of HPV are recognized, but not all of these types have the potential to cause lesions that may progress to malignancy. A hybrid capture technique (see above) is widely used to detect the DNA of 'high-risk' (HR) HPV genotypes in cervical brushings/washings, and there is evidence that women with minor cytological disorders can be excluded from colposcopy following a negative HR HPV result (Guyot *et al.*, 2003).

Monitoring Antiviral Therapy

In recent years, viral genome quantification to monitor the effect of specific viral therapy has become part of the clinical management of patients infected with HIV, HBV, HCV and those at risk of developing CMV disease (Berger and Preiser, 2002).

For HIV, regardless of the baseline viral load, a level of 1000 copies/ml has been found to be achievable in the majority of people by four weeks from start of highly active antiretroviral therapy (HAART). Failure to achieve this is strongly associated with failure to depress viral load below 50 copies/ml within 24 weeks (Gazzard *et al.*, 2006). Clinical trial data suggest that reduction of viral load to below 50 copies/ml predicts durability of antiviral response (Montaner *et al.*, 1998; Powderly *et al.*, 1999). Thereafter the purpose of regular monitoring of plasma HIV RNA levels is to monitor the success of therapy, and current protocols recommend subsequent tests at three to four month intervals (see Chapter 39).

Three- to six-monthly monitoring of HBV DNA level is an important tool in assessing response to antiviral treatment as most guidelines propose that suppression of HBV replication is a major therapeutic goal. It may be appropriate to use shorter monitoring intervals (every three months) for lamivudine monotherapy than for other nucleoside/nucleotide analogues because of the propensity for lamivudine resistance to arise (Valsamakis, 2007).

Evidence suggests that a lower baseline hepatitis C viral load predicts a more favourable response to combination therapy (pegylated interferon and ribavirin) for chronic infection (Yuki *et al.*, 1995), and that the required duration of treatment may be shorter (Shiffman *et al.*, 2007). Moreover, HCV RNA quantification has become vital for monitoring the response to therapy. In genotype

1 and 4 infections, if the HCV RNA load has not fallen 100-fold after 12 weeks of treatment, the likelihood of viral RNA remaining undetectable six months after completion of therapy, that is of achieving a sustained viral response (SVR), is very low (negative predictive value: 97–98%) and treatment should be discontinued (NICE, 2004). This leads to cost savings and a reduction in the inconvenience and side effects of treatment (Davis, 2002). Patients who become HCV RNA negative after only four weeks of treatment have the best chance of achieving SVR (as reviewed in Poordad *et al.*, 2008).

Virological monitoring of patients receiving anti-CMV therapy is important. Not only does a high viral load predict CMV disease in a number of risk groups such as solid organ transplant recipients (Fox *et al.*, 1995), HIV infected patients (Spector *et al.*, 1998) and congenitally infected newborn infants (Revello *et al.*, 1999), but persistent viraemia following onset of therapy or virological relapse on therapy is associated with continuing disease. Conversely, in stem cell transplant recipients treated with ganciclovir pre-emptively, clearance of viraemia can be an indicator to stop therapy (Einsele *et al.*, 1995). In all cases of antiviral drug monitoring using qualitative or quantitative molecular assays, a rebound in viral load or failure to suppress viral replication may reflect reduced drug susceptibility. In these cases, it may be appropriate to undertake drug susceptibility assays (see below).

Prediction of Transmission

It is reasonable to assume that a high viral load will predict a propensity to transmit infection. Studies on vertical HIV transmission suggest that the mother's viral load is a better indicator of the risk of vertical transmission than CD4 cell count (O'Shea *et al.*, 1998). The plasma HIV load has been shown to be the main predictor of heterosexual transmission in a study of HIV discordant couples in Uganda (Quinn *et al.*, 2000), and a high HIV load in genital secretions is also associated with efficient heterosexual HIV transmission (Chakraborty *et al.*, 2001). Mother-to-infant hepatitis C transmission is associated with a high HCV load (Dal Molin *et al.*, 2002) and similarly, in a study of 155 HIV and HCV co-infected women, the maternal plasma HCV RNA was significantly higher in those who transmitted HCV to their offspring than in those who did not (Thomas *et al.*, 1998). HBeAg has long been used as a surrogate marker of a high HBV virus load and therefore of high risk of mother-to-infant transmission in pregnant women; but increasingly studies are detecting significant levels of HBV DNA in HBeAg-negative individuals (Berger and Preiser, 2002). Following a number of incidents of transmission

of hepatitis B from HBeAg-negative health care workers (HCWs) to patients, guidance (HSC 2000/020) extended the role of HBV DNA monitoring in the United Kingdom to exclude HCWs with a DNA load of $>10^3$ genome equivalents/ml from practising exposure-prone procedures, whatever their HBeAg status. More recent guidelines allow HBeAg-negative individuals with a baseline DNA load of between 10^3 and 10^5 genome equivalents/ml to practice while on antiviral therapy if their DNA load is reduced to $<10^3$ genome equivalents/ml and is monitored under careful supervision every three months (Department of Health UK, 2007).

Viral Genetic Analysis of Transmission Events

Viral genome sequencing is now a standard method for studying transmission events. Relatedness between viruses is examined against a background of genetic variation ('viral quasispecies'). In such investigations, the choice of gene targets to amplify and sequence is important, and results must be subject to the correct statistical and phylogenetic analysis for reliable evidence of a transmission event. This approach has been particularly important in the investigation of HCWs infected with blood-borne viruses such as HBV (Nguai and Teo, 1997; Zuckerman *et al.*, 1995) and HIV (Blanchard *et al.*, 1998).

Sequence-relatedness between different virus isolates is also essential for virus classification. The data used to generate phylogenetic trees are usually derived from conserved genes, such as those coding for viral enzymes or structural proteins. This type of analysis has been used recently to develop a new classification of the *Retroviridae* (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_retro.htm).

Detection of Antiviral Resistance

Resistance has been documented for virtually all compounds with antiviral activity, and so the emergence of antiviral resistance in clinical practice should come as no surprise. Drug susceptibility depends on the concentration of drug required to inhibit viral replication and so drug resistance is not usually 'all or none', but relative. The genetic basis of resistance is becoming better understood, and specific viral genetic mutations have been associated with resistance.

As the use of antiviral drugs increases, there will be more pressure on diagnostic laboratories to provide assays to determine the causes of treatment failure, of which drug resistance is one. Laboratory assays for drug resistance fall into two major categories: phenotypic and genotypic. Their relative advantages and disadvantages are summarized in Table 1.8.

Table 1.8 Advantages and disadvantages of phenotypic and genotypic antiviral drug resistance assays

	Advantages	Disadvantages
<i>Phenotype</i>	Represents sum of all mutations Quantitative assessment of resistance (IC ₅₀ , IC ₉₅)	Expensive Labour-intensive Slow
	Can assess cross-resistance	Selection of culture-adapted strains (not with recombinant virus assay)
<i>Genotype</i>		
(a) Selective (e.g. point mutation assay, line probe)	Quick Relatively inexpensive (PMA) Semiquantitative (PMA)	Difficult to interpret single mutation in absence of other information
(b) Sequencing	Rapid Comprehensive information Background polymorphisms detected	Expensive Labour-intensive Expertise in genomic analysis required Simultaneous mutations not necessarily on same genome

Phenotypic Assays

Though phenotypic assays have been largely replaced by genotypic methods, the plaque reduction assay remains the gold standard for detecting HSV, CMV and VZV drug resistance. A specific titre (plaque-forming unit) of virus is inoculated on to a permissive cell monolayer, usually within a multiwell plate. These monolayers are overlaid with increasing concentrations of drug in a semisolid medium, thus preventing extracellular virus spread. The plaque reduction associated with drug inhibition can then be calculated, with results expressed as IC 50 or IC 90 (concentrations of drug required to inhibit virus production by 50 or 90%). Alternative methods for HSV include the dye uptake method, which quantifies viable cells within a viral-infected monolayer. The time-consuming nature of VZV and CMV culture techniques has led to the development of rapid culture methods, using viral antigen detection or genome detection to assess drug efficacy (Pepin *et al.*, 1992). All these assays produce different IC 50 values on the same isolates, and standardization is therefore required.

For HIV-resistance testing, recombinant virus assays have been developed. In these, a PCR product amplified directly from plasma virus is recombined with an HIV clone lacking the relevant gene (Hertogs *et al.*, 1998). The fragments can include the RT gene, protease gene and gag cleavage sites, and the resulting recombinant can then be screened for susceptibility to a range of drugs. Since the background clone of virus used grows rapidly in culture, this method is faster than conventional phenotypic assays.

With the development of CCR5 entry inhibitors such as Maraviroc to treat HIV infection, it has become important to be able to determine whether a potential recipient already harbours CXCR4-using (X4) viral quasispecies. Such viruses will be resistant to a CCR5 inhibitor drug and their increasing predominance in the viral 'swarm' is associated with more rapidly advancing HIV disease. The Trofile™ co-receptor tropism assay measures the ability of a patient's virus envelope gene to mediate entry into cells expressing CXCR4 or CCR5.

Phenotypic assays are clearly important since they reflect global determinants of drug resistance, but they require propagation of a virus stock before the assay is undertaken. This process is itself selective, and may lead to the final susceptibility assay being carried out on an unrepresentative species. On the other hand, phenotypic assays based on PCR suffer from the same genetic selection limitations as genotypic methods.

Genotypic Assays

An understanding of the genetic basis of drug resistance and the availability of automated and nonradioactive methods of nucleic acid sequencing have enabled widespread assessment of viral isolates with reduced drug susceptibility. With the increasing use of antiviral medication for a number of infections, most notably HIV and hepatitis B, these assays have become part of a routine diagnostic repertoire. Genotypic assays for drug resistance in CMV have also been developed (Bowen *et al.*, 1997) and mutations associated with multidrug resistance are now recognized (Scott *et al.*, 2007).

Sequence-based methods have largely replaced selective PCR (or point mutation assays, PMAs), line probe assays and RFLP assays for specific mutations associated with resistance. Recent advances in automated nucleic acid sequencing, such as the use of capillary sequencers, allow rapid high-throughput sequencing within a clinical laboratory setting. This has been most widely utilized for HIV and hepatitis B drug resistance assays, but it has other applications, for example the study of nosocomial transmission events. The biggest challenge of this technique is the manipulation and analysis of the data generated. Sequence editing and interpretation is required. With regard to HIV drug resistance, the identification of key resistance mutations depends on interpretation of variable drug susceptibility patterns and on the software systems utilized. When based on a product PCR amplified from the plasma, these techniques provide information only on the majority population within the quasispecies. They cannot exclude different mutations existing on separate genomes.

'Virtual phenotyping', a technique developed by Virco (www.vircolab.com), is a method of interpreting genotypic HIV resistance information with the aid of a large database of samples with paired genotypic and phenotypic data. By searching the database, viruses with genotypes similar to the patient's virus are identified and the average IC 50 of these matching viruses is calculated. This information is then used to estimate the likely phenotype of the patient's virus.

RECOMMENDED DIAGNOSTIC INVESTIGATIONS

Making an accurate virological diagnosis is critically dependent on receiving adequate specimens with information relating to the onset of symptoms and the clinical presentation. Swabs and tissue samples should be collected by trained staff and placed into virus transport media. If sample transport is delayed, samples should

be stored at 4°C or on wet ice (for a maximum of 24 hours). Assays for quantifying virus in blood require rapid specimen transport and appropriate processing and storage prior to analysis. It is the role of the clinical virologist to decide on the most appropriate investigations for any given clinical scenario. Laboratory request forms are important in this respect, and should encourage full documentation of clinical details. The practice of sending a serum sample to the virology laboratory with a request for a general 'screen' should be strongly discouraged. Instead diagnosis should be built upon the concept of syndromic presentation, its initial and continuing investigation, and appropriate management developing out of clinical progress, diagnostic findings and response to treatment. If the clinical presentation proves to be due to a virus infection, the virologist may have a leading role, but liaison with haematological, radiological, pharmacological and other clinical colleagues must be appropriate and continued.

Test Selection

Much of the work presenting to a virology laboratory is straightforward, taking the form of a particular screen (Table 1.9). But in other cases clinicians are presented with a patient whom they suspect may have a viral infection, yet they are unsure which tests to request. In some cases, they may request inappropriate tests. One of the most important roles of the laboratory staff, both technical and medical, is to assist the clinician in obtaining the correct diagnosis by choosing appropriate tests. In some cases sufficient clinical details on request forms will allow test selection in the laboratory (not necessarily the tests requested by the clinician!). In other cases, further information and possibly additional and different sample types will be needed. Many virology laboratories also provide serological testing for nonvirological infections, for example syphilis, toxoplasma and chlamydia (serological and nucleic acid-based testing).

Table 1.9 Examples of suggested screening assays for specified patient groups

Pre-stem cell transplant screen (donor and recipient)	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), anti-HCV, HBsAg, anti-HBcore, anti-HTLV 1, CMV IgG, EBV EBNA IgG, VZV IgG, anti-HSV, syphilis, toxoplasma-Ab
Renal dialysis patients	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), anti-HCV, HBsAg pre-dialysis Anti-HCV, HBsAg three-monthly. HIV repeat testing based on risk assessment
Antenatal screening	HIV (i.e. combined testing for anti-HIV 1 and 2 and for HIV 1 p24 antigen), HBsAg, Rubella IgG, syphilis

Table 1.10 Suggested testing strategy for the investigation of hepatitis and abnormal LFTs, with or without jaundice

First line	HAV-IgM, HBsAg and HBcore IgM, anti-HCV
Second line	EBV-IgM, CMV-IgM, HCV-RNA, hepatitis E IgM (any could be first-line, depending on history)
Third line	Dengue, yellow fever, leptospirosis, enterovirus, adenovirus and others depending on age, clinical details, travel history and so on

In the UK, the Health Protection Agency has proposed a number of national standard operating procedures (www.hpa-standardmethods.org.uk) that can help in suggesting a strategy for testing (e.g. VSOP 6: Hepatitis, jaundice and abnormal LFTs); but as illustrated in Table 1.10, the individual patient needs to be taken into account in order to select a cost-effective strategy for testing. There are also many situations where tests will be requested for which there is no evidence

base or recommendation to support testing, and many laboratories have developed brief clinical/educational comments explaining why such testing is thought not to be appropriate. Examples are given in Table 1.11. Clinical liaison leads to samples being used more appropriately, for example proposing hepatitis C antibody testing for a fatigued ex-drug user. Screening requests such as ‘TORCH’ should be discouraged in favour of test selection in response to specific clinical details. Testing for congenital and perinatal infection is complex, and for a neonate/infant is likely to require access to earlier stored samples such as the maternal antenatal booking sample or the infant’s dried blood spot taken at birth (the Guthrie card). Exposure to rash illness in pregnancy is very common (e.g. chickenpox, B19 virus) and national guidelines exist on rash illness and exposure to rash illness in pregnancy (www.hpa.org.uk/infections/topics_az/pregnancy/rashes/default.htm).

Table 1.12 illustrates some suggested first-line serological tests for common clinical requests in the United Kingdom. Selecting an appropriate repertoire of serological tests for a routine diagnostic laboratory and achieving an appropriate balance between testing samples in-house and sending samples to other laboratories

Table 1.11 Examples of comments for samples where serological testing may not be clinically indicated

Request	Comment
Chronic fatigue screen	Serological tests are of low value for investigating chronic fatigue. We are happy to discuss individual cases. Sample stored
Meningitis/encephalitis screen	Viral serology in the first week of meningitis or encephalitis is usually unhelpful. The sample has been stored. CSF is the best sample
?HSV infection	Serological testing is unlikely to be helpful. Please send appropriate sample (CSF, or swab of skin/mucosal lesion in viral transport medium) for viral culture ± PCR
Abdominal pain/diarrhoea. Viral screen please	There are no useful serological tests for viruses that cause primarily vomiting, diarrhoea or abdominal pain
Intrauterine death (IUD)/miscarriage with ‘TORCH’ screen requested	Serological tests are not routinely performed in cases of IUD/miscarriage unless there are clinical features suggesting a viral aetiology. If this is the case please contact the laboratory
Measles/mumps/rubella (MMR) screen	The assays that measure IgG to measles and mumps have not been validated for the determination of protection against these diseases: they were designed to assist in the diagnosis of acute infection. Therefore, it is not appropriate to use these assays for the purpose of excluding the need to give MMR
Atypical pneumonia/influenza	Testing acute samples for atypical and influenza serology does not contribute to acute patient management. Sample stored. If legionella infection is suspected, please send a urine sample for legionella urinary antigen

Table 1.12 Suggested first-line serological tests for common clinical requests in the United Kingdom

Clinical details	First-line tests
Arthralgia/joint pains	B19 IgM, HBsAg, Rubella IgM. Consider toxoplasmosis IgM if myalgia
Atypical pneumonia/influenza	Acute sample store. See above suggested comment. Testing of convalescent samples provides retrospective diagnosis and epidemiological data
Chronic fatigue	Samples not tested until discussed with clinician
Encephalitis	As indicated by clinical details. See HPA protocol QSOP 48
Endocarditis (culture negative)	Q fever, bartonella, chlamydia species, others as indicated
Glandular fever/lymphadenopathy	EBV-IgM, CMV-IgM, consider toxoplasma IgM and HIV test, bartonella if cat scratch a possibility
Hepatitis/abnormal LFTs	See Table 1.10 (also HPA VSOP 6)
Pregnancy and congenital infection—illness in the mother, abnormal fetal ultrasound findings or possible neonatal infection	Strategy depends on nature of illness, clinical findings and local protocols. ‘TORCH’ screening should be discouraged
Rash illness—maculopapular	Depends on local epidemiology. Consider B19, rubella, measles
Rash illness—vesicular	Depends on local epidemiology. HSV and VZV infections usually best diagnosed with a vesicle swab for PCR-based direct virus detection
Rash illness—exposure in pregnancy	See HPA protocols VSOP 33 and www.hpa.org.uk/infections/topics_az/pregnancy/rashes/default.htm

will depend on the size of laboratory, the population served, ease of testing and the throughput of tests. Many assays are best performed in batches, and in order to maintain an acceptable turnaround time it is not practical to provide a comprehensive repertoire in every laboratory. However, the increasing availability of multiple analyser systems that provide a wide range of cross-discipline immunoassays, random access facilities and automation allows even the smaller laboratory to offer a wide range of serological assays. This has to be balanced against the savings that can be made with economies of scale. In addition, obtaining a battery of immunoassays from a single commercial company may mean that compromises have to be made with certain tests not having the optimum sensitivity and specificity for the particular local population.

FUTURE TRENDS

In the light of rapid assay development and the continuing identification of new viruses, clinical protocols require constant updating. The era of retrospective viral diagnosis is over, replaced by rapid techniques which impact directly on clinical management. Competition for health care resources has meant that new techniques have replaced more traditional methods with limited clinical

value. Clinical virologists have to work closely with their clinical colleagues to establish new diagnostic criteria, develop protocols for use of antiviral drugs and monitor patients with persistent infections. The diversity of diagnostic methods now available makes communication between physicians and clinical virologists more important than ever before. The present multiplicity of traditional and new diagnostic techniques, as described above, suggests that a rationalization is now overdue. This will involve the abandonment of some older technologies, more discriminatory use of newer, especially molecular, ones, and technical collaboration with other pathology disciplines to improve efficiency and shorten turnaround times. Laboratories will need to embrace the electronic patient record (EPR) and remote requesting (‘order communications’), which, if well managed, will improve the quality of service provided by the laboratory, improving patient care. Laboratory information management systems will have to be fully integrated with the EPR in order to maximize the benefits from new information technologies.

A special emphasis must be placed on the needs of the immunocompromised patient population. They may experience life-threatening viral infections, which can present atypically. Ongoing antiviral prophylactic therapy may distort the nature and timing of presentation. Routine monitoring of transplant recipients is important

so that pre-emptive or early therapy can be initiated or immunosuppressive therapy modified as appropriate. Precise monitoring protocols will depend on the patient group concerned, availability of laboratory facilities and budgetary constraints. Nevertheless, in the context of high-risk patients such as those receiving long-term chemotherapy or transplants, the relative cost of virological investigations will be small. This population is constantly changing and expanding with new procedures and immunosuppressive agents, and the range of organisms to be monitored is likely to increase.

Alongside the increase in laboratory automation there is an increase in the integration of clinical virology with clinical microbiology laboratories. A step further is more extensive automation that integrates diagnostic immunoassay facilities across pathological specialities. This leads to economies of scale, though it may reduce the skill base and number of individuals trained in traditional virological techniques such as tissue culture and EM. Already, the distinctions between bacteriology and virology diagnostic techniques are becoming blurred, both providing opportunities for those skilled in molecular diagnostics and strengthening the practice of molecular diagnostics. With increasing automation in molecular techniques, the majority of general microbiology laboratories in the United Kingdom now offer at least a limited repertoire of molecular assays. For example, nucleic acid-based testing for *Chlamydia trachomatis* and other commercially-available assay systems are also being used for presurgery methicillin resistant *Staphylococcus aureus* (MRSA) screening and the detection of bacteria in sterile site samples by a PCR for 16S ribosomal RNA. Microbiology laboratories of the future may have to participate in bioterrorism surveillance for multiple infectious pathogens and collaborate in the rapid development of tests for new diseases of high social impact, as recently shown for the SARS caused by SARS-CoV (Raoult *et al.*, 2004).

There is a corresponding change in the training of medical staff in the United Kingdom, with broader based training across the infection specialities. There is a new cohort of physicians qualified to practice both as infectious disease clinicians and as laboratory-based staff. With possible future centralization and 'factory style' diagnostic testing facilities, laboratory-based specialists need to strengthen their role in clinical consultation and front-line management of patients, for example in hepatitis and HIV clinics and on transplantation ward rounds. For some, this broad-based training in infection demands a radical change in attitude and outlook.

We live in a global village, with rapid international travel and communication and the possibility of major environmental transformation due to climate change. Deforestation and expanding urban development facilitates

epidemic spread of infection by bringing human and animal populations closer together, allowing pathogens to 'jump' species. The threat of bioterrorism must not be neglected. These factors make human and animal populations more vulnerable than ever before to the epidemic spread of novel (as well as well-known) viruses, as seen in recent years with SARS-CoV and avian H5N1 influenza. In recognition of this, the World Health Organization (WHO), within the framework of the International Health Regulations, has developed a vision that '*every country should be able to detect, verify rapidly and respond appropriately to epidemic-prone and emerging disease threats when they arise to minimize their impact on the health and economy of the world's population*'. This should be achieved by focusing on three principles: contain known risks, respond to the unexpected and improve preparedness (www.who.int/csr/about/en/#strategy).

In rural, resource-poor areas of the world such as parts of South East Asia and Africa, little is known about the epidemiology of many viral infections. The burden of viral disease in such areas is likely to be considerable, but the infrastructure to implement the cross-sectional epidemiological studies necessary to define the causes of infectious disease is not available, largely due to economic constraints. Research in such resource-poor areas is fragmentary and often focuses on the interests of a specific individual or group. The developed world has been able to embrace the recent advances in diagnostic virology and improve clinical care, but in resource-poor areas basic diagnostic facilities which could go some way towards delivering the WHO vision are not available. For example, basic laboratory testing to support the roll-out of highly active anti-retroviral therapy (HAART) is often lacking in resource-poor settings. An important challenge for the infection community is to develop cost-effective and robust diagnostic and monitoring assays for use in the developing world. The discipline required to do this may also bring benefits by helping to rationalize the congested work schedules in laboratories in the developed world.

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