

# Molecular methods of measurement of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus infection: implications for occupational health practice

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## Abstract

**Over the past decade, several molecular techniques for the detection of human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) have been developed that have implications for occupational health practice. This review describes the techniques used for qualitative and quantitative detection of the viral genome, and briefly explains nucleic acid sequencing and analysis of phylogenetic trees. The review also discusses the current and potential uses of these techniques in investigations of transmission of bloodborne viruses by patient to worker and worker to patient, in the management of occupational exposure to blood, in research, and in the development of guidance and policy on infected healthcare workers who perform procedures prone to exposure.**

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Exposure to blood borne viruses continues to pose a serious occupational threat to healthcare workers (HCW). The viruses of greatest concern are hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV).<sup>1</sup> The use of universal precautions and the development and introduction of safer devices may have reduced the risk of exposure,<sup>2</sup> but the risks cannot be eliminated completely. Transmission of HBV, HCV, and HIV by infected healthcare workers to patients during procedures prone to exposure—that is, procedures where there is a risk that the patient may be exposed to the blood of the healthcare worker—is now well recognised. In consequence, the management of infected healthcare workers who perform procedures prone to exposure has become the subject of much debate.

The development of molecular assays for bloodborne viral infections has made it possible to detect acute infections at an earlier stage than would be possible with conventional techniques and to measure the concentration of virus present in blood during the acute or chronic phases of infection. Nucleotide sequencing followed by sequence comparison or construction of phylogenetic trees now allows genetic viral variation to be analysed in detail. This review explains the principles of these new

techniques and considers their possible applications in occupational health practice.

## Qualitative detection of viral genome: the polymerase chain reaction assay

Since the introduction of the polymerase chain reaction (PCR) in the mid-1980s, this assay has been used for the detection of various classes of infectious agents including a wide range of viruses.<sup>3</sup> It has proved most useful for the identification of viruses that are either completely unable to be cultivated or can be cultivated only with great difficulty. Infections in which antigen production is limited or in which serological responses are delayed or absent are also particularly appropriate for diagnosis based on the PCR.

Theoretically, any segment of DNA of known nucleotide sequence can be amplified by PCR. The sequence information is used to design synthetic oligonucleotide primers (generally 15 to 25 mers) which are complementary to opposite strands of the target DNA and separated by 20 to several hundred base pairs. A three step temperature cycling process is performed.<sup>3</sup> Firstly, the double stranded target DNA is denatured at 95°C to separate the strands. Next, the temperature is reduced to 30–60°C to allow annealing of the primers to the complementary sequence of each single stranded DNA. Finally, the temperature is increased to 72°C to allow the thermostable enzyme Taq DNA polymerase (from the bacterium *Thermus aquaticus*) to copy along both strands in opposite directions, thereby doubling the amount of the target DNA. The original strands and the newly synthesised strands both serve as templates for the next cycle. Repeated cycling generates an exponential increase in the number of molecules of target DNA. The amplified product can be reamplified with a second pair of internal primers (nested PCR), which has the advantage of increased specificity without the use of radioactive probes and the potential to detect minute amounts of target DNA. Amplification of ~10<sup>7</sup>-fold is obtained by 25–35 such cycles and even higher levels of amplification (up to 10<sup>12</sup>-fold) can be obtained with the nested primers. Although originally designed for DNA amplification, the technique can also be used to amplify RNA sequences if they are first converted into complementary DNA (cDNA) by reverse transcription (RT-PCR).

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## Quantitative measurement of the viral genome

### NUCLEIC ACID ASSAY

The PCR assay is so sensitive that it can detect a single molecule ( $\sim 10^{-18}$  g) of the sequence of interest, and large amounts of rare target DNA or RNA can be generated by amplification. However, it has been more difficult to measure the amount of target template present in the starting material.<sup>3</sup> The main constraint in obtaining quantitative data is inherent in the amplification process. As the amplification is an exponential process, small differences in any of the variables that control the reaction rate will dramatically affect the yield of the PCR product. Even when these variables are controlled precisely, there is sometimes a tube to tube variation that precludes accurate measurement. To overcome these problems and allow the precise measurement of specific target template in the starting material, a competitive PCR assay involving coamplification of a competitive mutated template (competitor) has been developed. The competitor uses the same primers as those of the target template but can be distinguished from the target template after amplification.<sup>4</sup> However, the competitive PCR method is labour intensive and is unsuitable for dealing with large numbers of samples. Methods more suitable for routine use are being introduced and evaluated.

### MEASUREMENT OF HBV, HCV, AND HIV: CURRENT PRACTICE

Several serological assays are used routinely to diagnose and monitor HBV infection. The presence of serum hepatitis B surface antigen (HBsAg) indicates a current (acute or persistent) HBV infection, whereas hepatitis Be antigen (HBeAg) serves as a marker for active viral replication.<sup>5</sup> However, the absence of HBeAg in blood does not exclude viral replication, and the concentration of serum HBV DNA may be a more accurate reflection of replicative activity of HBV than HBeAg.<sup>6</sup> Several types of assay for measuring serum HBV DNA concentrations have been developed and are commercially available. These include a liquid hybridisation assay (Abbott Laboratories, North Chicago, IL, USA); an RNA-DNA hybrid assay (Digene Diagnostics, Silver Spring, USA); a branched DNA signal amplification (bDNA) assay (Quantiplex HBV-DNA Assay, Chiron, Emeryville, CA, USA) assay and the nucleic acid based crosslinking assay (NAXCOR, Menlo Park, CA, USA).<sup>7, 8</sup> These assays have varying levels of sensitivity (1–5 pg/ml, which corresponds to 0.28 to 1.4  $\times 10^6$  copies/ml) and, because of a lack of standardisation, interassay comparisons have shown poor agreement.<sup>7, 8</sup> Until the assays have been properly standardised, any comparison of viral genome concentrations between cases should be made in the same laboratory on the same test run of the same assay; other comparisons should be interpreted with caution.

An ideal candidate for PCR based detection methods is HCV because at present HCV cannot be isolated reliably from clinical specimens

by culture, and commercial serological assays for the detection of HCV antigen are not yet available. Thus, the presence of viral genome and its concentration can be detected only by molecular assays. As well as in house competitive RT-PCR, two commercial assays (HCV Monitor (Roche Diagnostics) and Quantiplex HCV RNA 2.0 (bDNA 2.0)) are available for the measurement of HCV RNA.<sup>9</sup> The detection limit of the bDNA 2.0 assay is  $2 \times 10^5$  copies/ml, and the cutoff of HCV Monitor is about 1000 copies/ml. Previous versions of both assays underestimated HCV RNA concentrations in patients with HCV genotype 2 and 3 infection; the current versions have been redesigned to allow equivalent measurement of all common genotypes.<sup>9</sup> Again, comparisons between assays show poor agreement, and the same provisos as for HBV apply.

Although quantitative culture of HIV is possible, it is time consuming, cumbersome, and requires appropriate laboratory safety equipment. Measurement of p24 antigen with enzyme immunoassay (EIA) can be performed quickly, without special equipment, but is of relatively poor sensitivity, and therefore of limited value. Three different commercially available plasma HIV RNA assays are currently used for the measurement of HIV viral load.<sup>10</sup> The bDNA (Quantiplex, Chiron) is a signal amplification assay based on sequential oligonucleotide hybridisation steps and measured by comparison with an external standard, whereas the Amplicor HIV monitor assay (Roche Diagnostics) and the nucleic acid sequence based amplification (NASBA, Organon Technika, Boxtel, The Netherlands) are based on a target amplification technique, and use an internal standard for measurement. The correlation between the three assays for HIV RNA measurement is, as for HBV and HCV, relatively low, and the same constraints on comparisons of test results apply.

### DNA sequencing and molecular evolutionary analysis

Sequencing of DNA refers to the enumeration of individual nucleotide base pairs along a linear segment of DNA. The method of DNA sequencing has evolved considerably since the first techniques were described in the middle of the 1970s. Two procedures were initially developed. In the method of Maxam and Gilbert, the fragments are derived from the original target DNA by its chemical cleavage at one or two bases. By contrast, Sanger's method uses the incorporation of dideoxynucleotide analogues for the specific ending of the enzymatic copying of the target DNA. The fragments are then separated according to size by electrophoresis, and the sequence of the original target. Although the chemical cleavage method is still used in research laboratories, the technical advantages of dideoxy sequencing make it the method of choice for most tasks. Single strand or double strand DNA generated by PCR can be used directly for DNA sequencing, which can now be performed as part of the actual PCR, in a process known as cycle sequencing. Automation has made it possible to double the

lengths of readable DNA sequence obtained after a single sequencing run, to over 400–800 base pairs. The instruments in use at present rely on variations of non-radioactive fluorescent labelling, PCR, and gel electrophoresis.

Molecular evolutionary analysis is a statistical tool used to explore trends in virus evolution. As a virus evolves, changes in nucleotide sequence occur. A mathematical model is needed to estimate the mean number of nucleotide substitutions, and must be designed to account for all possible combinations of nucleotide substitution and the speed with which substitutions can occur. The model, and the knowledge of the number of nucleotide differences between the strains of virus of interest, is used to work out the phylogenetic relation between them and to construct a phylogenetic tree.

Comparison of nucleotide sequences of viral genomes derived from different patients can be used to help to determine whether an infection in a recipient patient is linked to an infection in a donor. DNA fingerprinting, or sequence homology comparison, has been used to study the pattern of transmission of DNA viruses including HBV. It is based on the assumption that the variable region of DNA viruses is variable enough to differentiate between different sources but that DNA viruses evolve slowly, so that major differences between strains are more likely to be accounted for by differences in source than by within-host evolution. It is known that populations of closely related viral genomes (quasispecies) exist in patients with chronic viral infections, including HBV, HCV, and HIV. Studies have shown that a minor virus clone from a donor may prevail as the dominant species in a recipient, possibly by selection after transmission. When sequencing is performed directly on DNA derived from amplification by PCR, the predominant clone in a given host will be identified but minor clones that make up <10% of the viral population may be overlooked. Thus, unless the amplified PCR products are cloned and subsequently sequenced separately to identify minority quasispecies, genuine transmission events may be missed.

RNA viruses, including HCV and HIV, evolve much more rapidly than DNA viruses. This means that, when studying HCV or HIV transmission, information provided by nucleotide sequencing may not be sufficient to determine whether two strains of virus are unrelated to each other. Supplementary information about divergent time, estimated from a phylogenetic tree constructed by molecular evolutionary analysis, is used to augment the information provided by nucleotide sequence fingerprinting.

#### **Implications for occupational health practice**

Molecular techniques for genome detection and quantification have potential applications in several areas of occupational health practice. These include the management of occupational exposures to blood and body fluids; the management of healthcare workers infected

with HBV, HCV, or HIV; and the investigation of possible transmission of a bloodborne virus either to, or from, a healthcare worker.

#### **MANAGEMENT OF OCCUPATIONAL EXPOSURES TO BLOOD AND BODY FLUIDS**

In general, the risk of transmission associated with percutaneous exposure (through an injury with a contaminated needle or other sharp object) is greater than that associated with mucocutaneous exposure to the same source. Similarly, exposure to blood from an infected source is of greater risk than exposure to other body fluids. Follow up studies of exposed healthcare workers have provided basic information about the risks of transmission from a single percutaneous exposure to blood from an HBV, HCV, or HIV infected source. These studies suggest that the risk of infection after exposure to a source of HBeAg is around 30%<sup>11</sup>; after exposure to a source of HCV around 2% (range 0%–10%),<sup>12–14</sup> and around 0.3% after exposure to HIV.<sup>15</sup> There is indirect evidence that the level of viraemia in the source is one of the factors that determines transmission risk. For example, in HIV infection (in which viral titres increase as the infection progresses) most reported cases of documented seroconversion after occupational exposure have followed exposure to a source patient with AIDS rather than asymptomatic HIV infection.<sup>16</sup> Also, exposure to blood from a source patient in the terminal stages of HIV infection was identified as increasing the risk of seroconversion in a recent case control study of exposed healthcare workers.<sup>17</sup> So, although the overall risk of infection after a percutaneous exposure to HIV infected blood is around 1/300, for some exposures the risk will be higher, and for some, lower. Incorporation of newer techniques for measurement of HIV and HCV RNA concentrations in source patients into follow up studies of exposed healthcare workers offers the possibility of refining these estimates of risk. This is despite the fact that plasma HIV RNA measurements reflect the concentration of cell free virus, rather than cell associated virus, in peripheral blood.

This would allow clinicians to provide healthcare workers with better information, and might eventually allow recommended follow up schedules and prophylaxis regimes after exposure to be tailored more precisely to specific exposures.

Usually, it is recommended that healthcare workers who report a considerable occupational exposure to a source known to be infected with HIV or HCV should be tested for anti-HIV or anti-HCV (as appropriate) at 6, 12, and at a minimum of 24 weeks after exposure.<sup>18</sup> Here, tests based on the PCR have two possible uses. Firstly, they may be used to provide earlier confirmation of suspected infection (in, for example, a healthcare worker who develops symptoms compatible with acute retroviral syndrome) by enabling infection to be detected at the stage when the worker is viraemic but has not yet produced detectable antibody. Secondly, their integration into the follow up schedule as well as antibody testing

may, if results are negative, give the healthcare worker additional reassurance that infection has not occurred. Their use for this purpose, however, does not allow the period of follow up to be shortened, as a negative result of a PCR test at 3 months after exposure does not preclude detection of seroconversion at 6 months after exposure.<sup>16</sup>

Tests for genome detection based on the PCR may also be useful when dealing with an exposure to a source patient who is known to be at high risk of infection, but who tests anti-HIV or anti-HCV negative. The test results may enable the clinician to distinguish an infected (and infective) source from a source that, because viral nucleic acid is undetectable, is likely to pose a much lower risk. At the moment, these tests are relatively expensive, and are most used by referral centres. If, in the future, the tests become cheaper and are used more widely by diagnostic laboratories, there would be an argument for using them to test all identifiable source patients, as patients' responses to direct questioning may be an unreliable indicator of risk of infection.

Seroconversion despite zidovudine prophylaxis after exposure to HIV is well recognised,<sup>16</sup> and it can be only a matter of time before HIV seroconversion despite prophylaxis with a combination of three antiretroviral drugs occurs. When this happens, it will be important for future recommendations on antiretroviral prophylaxis to determine, with these newer techniques, whether the development of resistance to antiretroviral drugs used in the treatment of the source could have contributed to the failure, or whether some more fundamental mechanism underlies transmission.

#### MANAGEMENT OF INFECTED HEALTHCARE WORKERS

Clinicians who provide occupational advice will not normally be primarily responsible for the medical management of infected healthcare workers, but need, nevertheless, to have an understanding of the uses of newer techniques in monitoring responses to antiviral treatment, and in determining progression of disease (table).<sup>19</sup> For example, the concentration of HCV RNA seems to be a useful predictor of response to antiviral treatment<sup>20</sup> and recent studies have shown that measurement of plasma viral load represents the most accurate long term prognostic marker for HIV infection, independent of CD4+ cell count. A single plasma RNA measurement is suggested to predict clinical events occurring 10 years later.<sup>21</sup> Also, these assays are used to monitor the

Table 1 Relevance of measurement of viral genome at work of hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infection

	HBV	HCV	HIV
Measurement of infectivity	+++	++	+
Early diagnosis of acute infection	+	++	++
Progression of infection	+	-	+++
Predictor of antiviral treatment	+++	+++	+++
Resolution of unusual serological profiles	++	++	+

+++ Very high relevance; ++ high relevance; + limited relevance; - no relevance.

response of infected patients to antiretroviral treatment.

It is now generally accepted that healthcare workers infected with HBV, HCV, or HIV may transmit infection to patients during procedures prone to exposure. Most healthcare workers do not perform procedures prone to exposure, and, provided that they adhere to standard infection control techniques, present no risk to patients. There is as yet no international consensus on the extent to which the practice of infected healthcare workers (primarily surgeons and dentists) who perform procedures prone to exposure should be restricted. In the United Kingdom, healthcare workers who are infected with HIV or HBV and who have detectable serum HBeAg may not perform procedures prone to exposure.<sup>22-23</sup> Those who are infected with HCV, or who are infected with HBV but who do not have detectable HBeAg may, for the moment, continue to practice unrestricted in the United Kingdom unless they have been associated with transmission to patients. In the United States, recommendations on restricting practice of healthcare workers infected with HBV are also based on the presence or absence of serum HBeAg.<sup>2</sup> However, six transmission incidents involving HBsAg positive, HBeAg negative surgeons have now been reported from the United Kingdom,<sup>24-26</sup> and a seventh is being investigated. Nucleotide sequencing of HBV DNA from the surgeons and from the infected patients was used to confirm transmission. It was also used to examine the strains of HBV involved; all six surgeons were carriers of HBV with a nucleotide substitution in the precore region of the viral genome, at codon 28. This base change encodes a premature stop codon, which prevents the expression of HBeAg but allows the continued replication and assembly of infectious virus in the presence of anti-HBe. The HBV DNA was detectable by PCR methods in all six surgeons, although at much lower concentrations than in HBeAg positive control samples. It seems likely that current United Kingdom guidance will be amended, and that, in the new guidance, recommendations on restricting practice of healthcare workers who are HBsAg positive but HBeAg negative will be based on serum HBV DNA concentrations as measured by amplification based assays. As yet, no country has introduced guidance which prospectively restricts the practice of healthcare workers infected with HCV. The ability to measure HCV RNA concentrations in anti-HCV positive people might enable any future recommendations to define HCV infectivity in terms of HCV RNA concentrations, rather than on the simple presence of anti-HCV.

Use has been made of newer molecular techniques in all of the recent investigations in which transmission of a bloodborne virus from an infected surgeon to a patient has been documented.<sup>24-29</sup> They have allowed investigators to ascertain the source of infection when a single infected patient has been identified,<sup>24-28</sup> and have also allowed infected healthcare workers to be excluded as the source of a patient's infection.<sup>30-31</sup> Techniques for comparison of

viral genomes may also be helpful in deciding the source of an infection supposedly acquired occupationally, particularly where a healthcare worker has sustained multiple occupational exposures, or has non-occupational risks of blood borne virus infections.

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