MOLECULAR BIOLOGY: APPLICATIONS IN TRANSFUSION MICROBIOLOGY

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Introduction

Detection of microbial infections in blood donors is a key task in blood transfusion practice. A blood transfusion provides an ideal portal of entry to a host (the recipient) for those infections (e.g., HBV, HCV, HIV, HTLV, CMV, malaria and Chagas' disease) whose persistence renders them a significant risk for transmission via plasma or cellular components. Since many such infections are due to viruses, prevention rather than treatment is the obvious goal. Classically, microbiologists have detected the presence of an infectious agent by culture, animal infectivity or serological techniques. In the context of the requirement for rapid and specific screening of large numbers of blood donations, culture and infectivity assays are patently inappropriate, even if such techniques were available: for the majority of transfusion-transmitted infections (TTIs) they are not. Microbiologists have therefore had to rely heavily on serological techniques to detect antibodies to the agents. With the persistent agents characteristically transmitted by transfusion, this approach is workable because the presence of specific antibody signals not just previous cleared infection, but often continuing infectivity. Nevertheless, microbiologists have long hoped for effective methods for direct detection of the agent, or at least an indication of the presence of the microbe rather than just the immune response to it. With the advent of molecular biological techniques to detect microbial genomes, transfusion microbiologists are now in a position to assay nucleic acids, providing a powerful tool for seeking the agent itself. This technology has the potential for revolutionising our practice, but as with all advances, it brings with it its own problems and pitfalls and these will be described. A basic understanding of the principles behind the most commonly used techniques for DNA amplification(1) (polymerase chain reaction or PCR) will be assumed.

Serology vs Molecular Biology

The principles of serological tests using specific antibodies to detect microbial antigens or specific antigens to detect microbial antibodies have been described.(2) Serological techniques are generally simple, rapid, economical and relatively sensitive. However, the presence of antibody, even in potentially persistent infections, does not always signal infectivity. Conversely, infectious virus may be present for several days or weeks (depending on the agent) prior to the appearance of detectable antibody; this will be illustrated in more detail later. False-positive reactivity due to cross-reactions or to contaminants in the coating antigens may also pose problems for serological assays. In contrast, detection of nucleic acid after amplification will enhance sensitivity of detecting infectivity in an infection prior to seroconversion. Furthermore, provided contamination of the sample to be tested is scrupulously avoided, choice of appropriate primer pairs should offer a test system which is potentially more specific than serological techniques.

Detection of amplified nucleic acid is, however, not devoid of potential problems, as follows:

1. False-positivity

Despite the potential for better specificity than serological assays, the very sensitivity of tests such as PCR make them highly susceptible to false-positivity from cross-contamination. This has been clearly demonstrated in the 'Eurohep' studies on the comparison of PCR performance in a range of European laboratories[3] where only a minority were able to obtain completely correct results on a standard panel of samples. Cross-contamination may involve other test samples, for example when the same probe is used to aspirate samples in automated sampling systems (even when these are washed between samples), or be due to the potential for splash contamination when samples are being uncapped and centrifuged in the context of a busy blood centre processing hundreds of samples daily. Samples may also be contaminated by previously amplified products, which is why amplification and DNA detection should be performed separately (preferably in a different room) from sample preparation. A method to overcome product contamination problems will be described later.

The choice of primers is also important to avoid detecting related agents sharing conserved nucleic acid sequences. This may be particularly relevant with the flavivirus-like viruses. On the one hand, an assay for HCV DNA should be capable of detecting all of the major types and subtypes but on the other hand detection of related (but distinct) viruses such as the recently described hepatitis G virus[4] may cause confusion. This issue, and the actual practical relevance of this potential problem, will become clearer as more data on viruses such as HGV becomes available.

2. Complexity

Detection of amplified nucleic acid is a more demanding technique than serology. The latter has been refined over many years whilst the former is still in its relative infancy. For optimal sensitivity, nucleic acid extraction should be as efficient as possible. With RNA containing viruses, the stability of the nucleic acid has to be taken into account and transcription to DNA is itself a relatively inefficient process. The need for transcription can be avoided by the use of NASBA (nucleic acid sequence based amplification), which is an alternative technique to PCR and will be described later. As yet, the degree of automation in molecular biological testing is much inferior to what is achievable for immunoassays (e.g., the Abbott PRISM totally automated chemiluminescent immunoassay). Nevertheless, microplate-based detection systems for PCR are now routinely available (see below), and automated systems are under development. However, nucleic acid detection remains a more time consuming and labour intensive process than serological techniques.

3. Cost

Nucleic acid detection is considerably more costly than serology and, unless this extra cost is reduced, the impact on mass screening budgets would be enormous. Although the testing of pools of samples would provide cost savings, this would involve extra complications, demand effective sample identity tracking technology and would

have to be balanced against the concomitant loss in sensitivity in terms of the individual samples.

4. Small Test Volume

Compared with the volume of a whole blood donation, the test sample is several orders of magnitude lower; on a statistical basis, genomes may be undetected although present in low numbers in a blood donation and at a level capable of transmitting infection. Therefore, although exquisitely sensitive, even techniques such as PCR cannot be guaranteed to detect every infectious unit of blood.

5. Detection of Non-Infectious Nucleic Acid

PCR may detect sequences of nucleic acid even if these are from incomplete or nonviable genomes. Virally inactivated blood products may therefore test PCR positive, even though they may be non-infectious.

Potential Applications of Nucleic Acid Detection in Transfusion Microbiology

These may be summarised as follows:

1. Detection of virus in serologically negative ‘window’ periods of infection.
2. Detection of virus in pools of plasma.
3. Assisting in confirmation of microbial reactivity.
4. Determining infectivity as opposed to immunity in seropositive samples.
5. Detection of variants/mutants, using appropriate primers.
6. Amplifying genes for sequencing.
7. Tracking infection (epidemiology).
8. Genotyping and speciating genomes.

These potential applications will now be considered in more detail.

Detection of Virus in ‘Window’ Periods

HIV infections serves as a good model to illustrate this application. Estimates from published data[(5)] are depicted diagramatically for the HIV ‘window’ period in Figure 1.

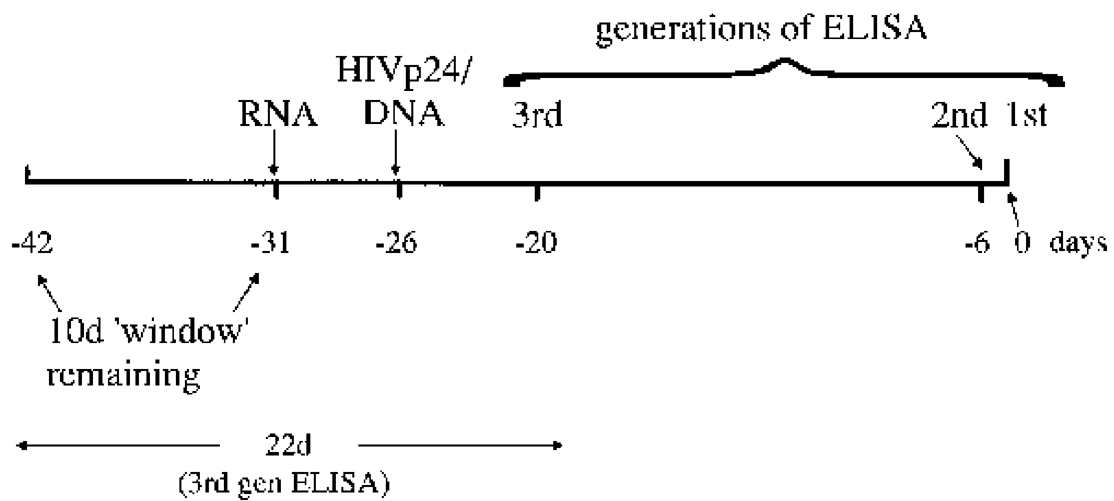


Figure 1. HIV ‘window’ period.

Time 0 is when first generation anti-HIV ELISAs would score positive during the course of HIV seroconversion. On average, second generation assays would react positive 6 days earlier than first generation tests, but third generation ‘sandwich’ assays detect antibody almost 3 weeks earlier (presumably because they can detect IgM as well as IgG classes of antibody). This leaves a residual ‘window’ period from the time of infection of approximately 3 weeks with such assays. Detection of HIV RNA after reverse transcription to generate DNA, which is then amplified by PCR, leaves a 10 day residual ‘window’. Direct detection of HIV DNA incorporated into the genomes of host white cells provides a gain of only 6 days compared with third generation anti-HIV ELISA, and HIV p24 antigen would be detectable at the same time as HIV DNA. Another example of the reduction in the ‘window’ period by PCR is afforded by HBV infection (Figure 2).

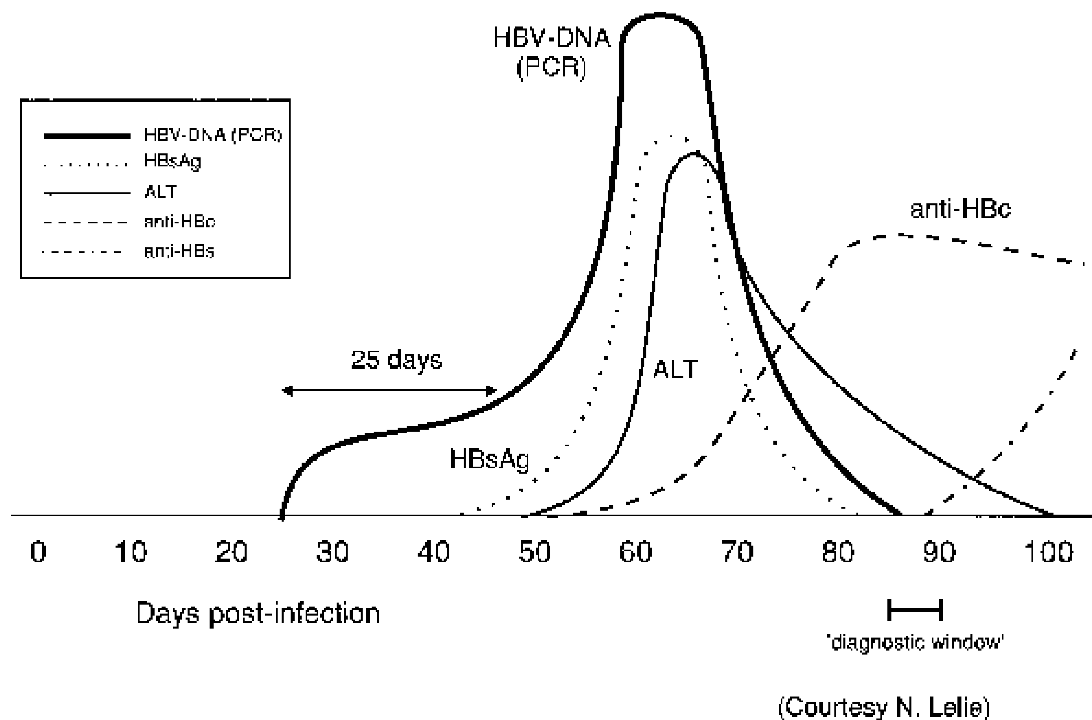


Figure 2. HBV seroconversion window

In the case of HBV, however, the extra detection span provided by PCR is longer than with HIV because of the generally longer incubation period to the development of detectable markers.

Some explanation of the use of the term 'window' period is warranted at this point. The term 'window' period was originally applied to the period in an acute HBV infection subsequent to the disappearance of detectable HBsAg and prior to the appearance of anti-HBs (Figure 2). This gap is, however, sensitively 'covered' by testing for anti-HBc. Currently, 'window' period is also applied when using antibody detection tests and refers to that period following infection, but prior to the development of antibodies, when infectious agent may be present in donor blood: in the case of the HBsAg carrier state, there is also a period (Figure 3) with some carriers, usually many years after infection, when HBsAg may be undetectable, but blood (especially the large inoculum afforded by transfusion) may still be infectious. Although HBV DNA may or may not be detectable, anti-HBc at high titre will be present.

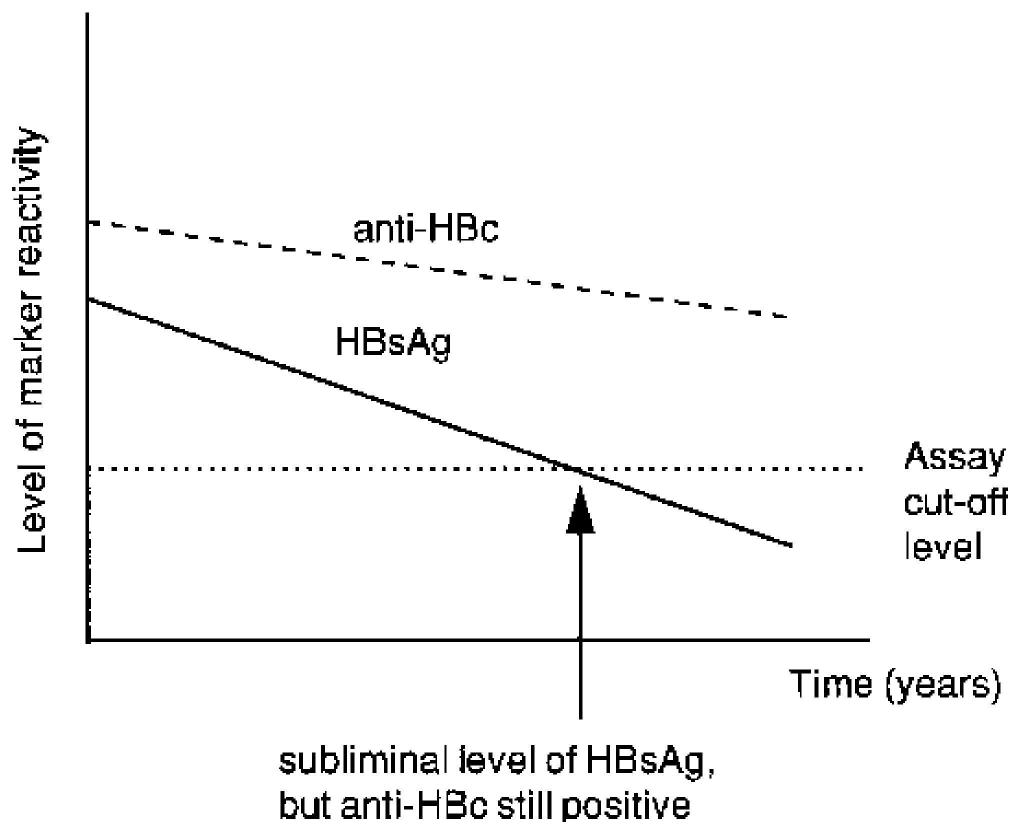


Figure 3. Anti-HBc in 'tail-end' carriers of HBsAg.

Detection of Virus in Pools of Plasma

The exquisite sensitivity of PCR can facilitate detection of a viral genome from a single contaminating plasma donation even after it has been pooled with as many as 10,000 other donations. This has led to moves to PCR test plasma pools prior to fractionation as an extra safety measure. Nevertheless, a positive PCR reaction does not necessarily relate to infectious virus being present. An economical approach to the use of PCR for testing pools and sub-pools of samples to detect virus has also been suggested.(7)

Assisting in Confirmation of Microbial Reactivity and Determining Infectivity as Opposed to Immunity

For most serological screening of blood donors, reproducibility of reactions in alternative assays of equivalent sensitivity, together with immunoblot procedures where appropriate, provides reliable confirmation of infection. For HBsAg reactivity, neutralisation and anti-HBc testing is also available. However, genomic detection can sometimes provide valuable additional evidence of infection, especially when blotting

assay results are indeterminate. A positive PCR will confirm infection, but a negative result leaves open the slight possibility of very low level viraemia, although this is usually very unlikely. In general, detection of viral genomes in a seropositive sample confirms continuing infectivity as opposed to immune clearance.

Detection of Variants and Mutants

Provided that highly conserved primers are used, mutant or variant strains that might test negative on certain marker assays should be detectable by PCR. Usually alternative immunoassays will be more conveniently applicable than PCR for routine screening. For example, the surface antigen mutants of HBV[8] that are not detected by certain monoclonally based HBsAg assays can be detected by appropriate PCR tests and also by alternative HBsAg assays employing antibodies against other HBsAg determinants.

Amplifying Genes for Sequencing, Genotyping and Tracking Infection

When PCR is used to amplify a fragment of microbial gene, nucleotide sequencing is facilitated. Epidemiological tracking of infection is thus possible at a molecular level to provide definitive proof of the spread of infection.[9] For example, in cases of putative sexual, materno-foetal, needlestick and transfusion transmission, the exact genetic identity of small peculiarities of individual isolates can be determined, thereby often providing conclusive evidence of transmission. Strain-specific restriction endonuclease sites can also be exploited for differentiation of viral strains.

The Range of Molecular Detection Techniques

Early nucleic acid detection was based on hybridisation techniques in liquid or solid phase whereby labelled nucleotides could bind to the nucleic acid sequence to be detected. This method can be enhanced by linear amplification of the reaction as in the branched-DNA (bDNA) technique, which employs an amplifier with 15 branches and each branch can bind three signal molecules. However, even these modifications do not allow the sensitivity of such systems to match the geometric amplification of methods such as PCR or NASBA, both of which have similar sensitivity.

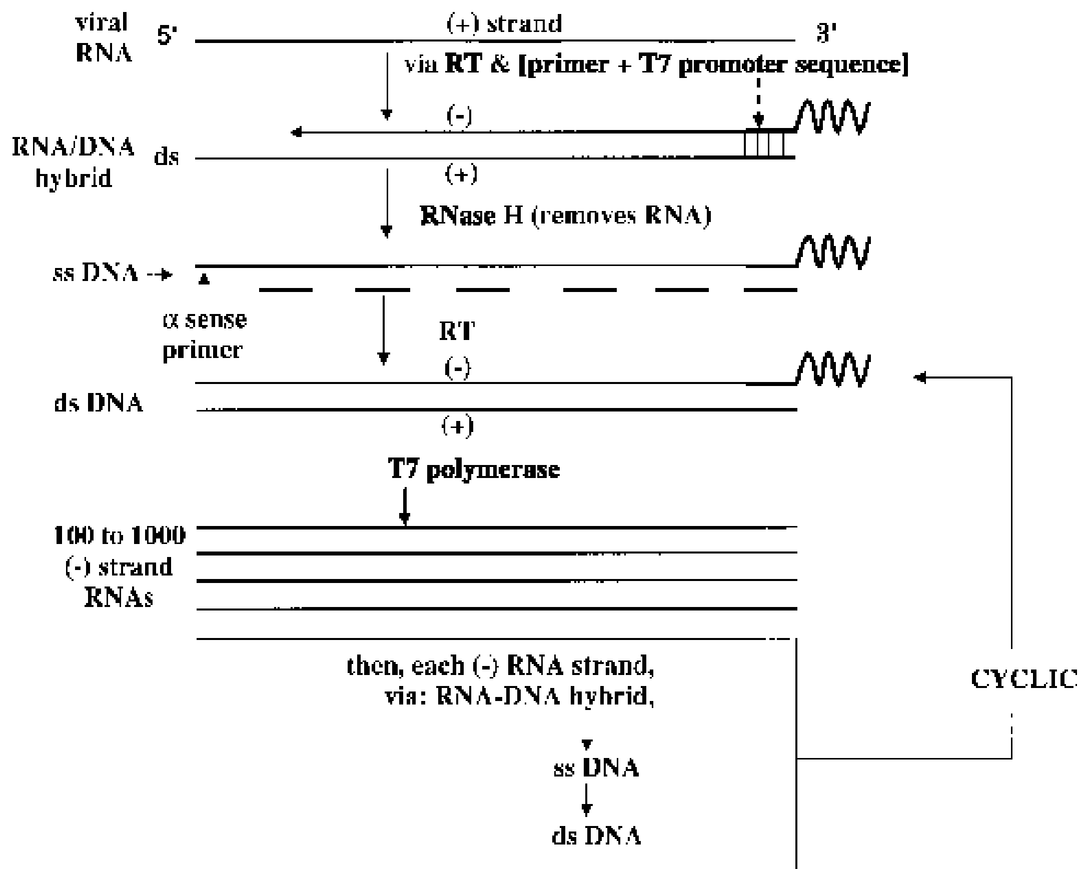


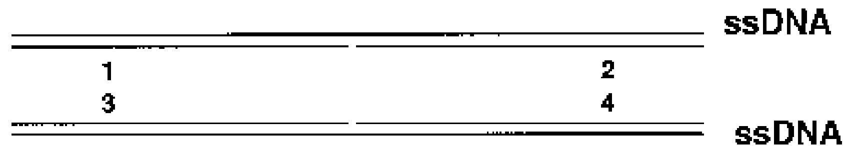
Figure 4. NASBA method.

The basis of the NASBA technique is outlined diagramatically in Figure 4. Unlike PCR, amplification of RNA is effected and no thermal cycling is involved: instead, the method is based on the natural mechanism of bacteriophage replication which requires the attachment of a bacteriophage T7 promoter sequence onto the minus strand of a double stranded DNA. The first stages of the NASBA process are therefore designed to produce such a molecule. Thereafter, the enzyme T7 polymerase directs the production of 100 to 1,000 minus strand copies of RNA. Each of these can then themselves be amplified in a cyclic repetition of the earlier stages. Finally, the amplified RNA is detected in an enzyme-linked gel assay by hybridising it to a nucleic acid probe bound to horseradish peroxidase. The product is visualised via an enzyme substrate. Alternatively, an electroluminescent label can be used for quantitative luminometry.

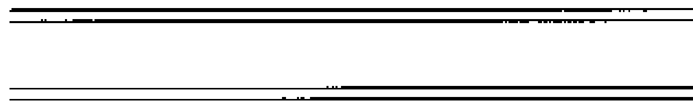
Several other amplification techniques and modifications have been developed. These include the ligase chain reaction for amplification of DNA (Figure 5). In this method, the target DNA is denatured and four complementary oligonucleotides are hybridised to the two single-stranded DNA molecules. The ligase enzyme then links adjacent complementary nucleotides to provide a continuous strand. The product is then denatured and the process is repeated cyclically.

LIGASE AMPLIFICATION OF DNA

1. **denature target DNA**
2. **hybridise 4 complementary oligonucleotides at 65°C**



3. **ligase attaches *adjacent* complementary nucleotides to 'fill the gaps'**



4. **denature and continue cycling → exponential amplification**

Figure 5. Ligase chain reaction.

Commercially Available PCR

The basis of PCR is now well known and will be assumed to be familiar to the reader. Sensitivity and specificity can be enhanced by use of 'nested' PCR[1] when the product of the first round of amplification is subjected to a second round of amplification using 'inner' primer pairs that locate within the sequence defined by the original 'outer' primer pairs.

Commercial assays such as the 'Amplicor' kits for detection of HCV and HIV nucleic acids are now available. In these assays, the DNA polymerase enzyme Thermus aquaticus is replaced by a similar enzyme from another thermophilic bacterium Thermus thermophilus. This enzyme has the convenient property of combining reverse transcription and DNA polymerase activities in the same molecule, making the system especially convenient for detecting RNA virus nucleic acid. The kit also employs the enzyme uracil-N-glycosylase (UNG). This enzyme degrades uracil, which replaces thymine in the deoxynucleotide triphosphate mixture of the Amplicor formulation. Therefore, prior to reverse transcription and the polymerase chain reaction process, UNG is incubated with the reaction mixture at 50¡C to destroy any contaminating product of previous amplification procedures that may have been introduced into the test system. This technique allows the avoidance of false positivity due to laboratory contamination of sample by previous amplification procedures. Cross-contamination by native extraneous DNA is, however, not eliminated, so meticulous adherence to procedures for avoidance of sample contamination is still required. For visualising the product of amplification, the use of agarose gel electrophoresis incorporating ethidium bromide, which intercalates

with DNA to fluoresce under UV light, has been superseded by biotinylation of one of the primers. This allows avidin conjugated to horseradish peroxidase to bind to the amplified product and produce a colour change, with appropriate substrate, in microwells. These colours can be measured automatically in a spectrophotometer. Following nucleic acid extraction, the procedure takes six hours for completion.

Sensitivity of Different Systems

The basal sensitivity of PCR can be enhanced not only by nesting, but also by enhancing nucleic acid extraction (e.g., with guanidinium isothiocyanate) or by monoclonal antibody capture of virions prior to nucleic acid extraction. For HBV this has been reported by Liang et al to allow detection of < 10 virions/200ml of sample.[(10)]

In general, the following levels of HBV DNA can be detected by the various methods:

	genomes/ml
PCR	2.5×10^2
bDNA	2.5×10^6
hybridisation	2.5×10^7
membrane hybridisation	2.5×10^7
	(courtesy, Dr Zaaijer)

In 80 anti-HCV ELISA and RIBA positive samples, 89% were Amplicor positive, 90% were ‘in house’ PCR positive (Central Laboratory, Amsterdam) and only 68% were bDNA positive. With HBV, the differences of viral load in HBeAg positive vs negative carriers of HBsAg, with different assays, is shown in Figure 6. Viral load for different agents[(6)] is shown in Figure 7.

HBV-DNA assays	%HBV-DNA +ve		
	HBsAg +ve		HBsAg -ve
	eAg +ve (n=30)	eAg -ve (n=79)	(n=35)
PCR	100%	90%	0%
bDNA	73%	25%	0%
Hybridization	67%	13%	0%
Dot blot	40%	8%	0%

Figure 6. Performance of four HBV-DNA assays. (Courtesy Dr. Zaaijer)

Virus	genomes/ml	
	window infection	sero positive carrier
HIV-RNA	$10^7 - 10^8$	$10^3 - 10^6$
HCV-RNA	$10^8 - 10^9$	$10^3 - 10^7$
HBV-DNA	$10^4 - 10^5$	$10^3 - 10^9$

Figure 7. Virus concentration in window infection. (Courtesy Dr. Zaaijer)

Conclusion

Molecular techniques have greatly enhanced our understanding of transfusion microbiology. A rich range of techniques exist, each with their advantages and disadvantages. However, despite the enormous amplification of viral genome (represented by the large icosahedron) the volume of test samples is minute compared with the volume of a blood component, and the recipient remains the most sensitive measure of microbial infectivity.

References

1. Barbara JAJ, Garson JA: Polymerase chain reaction and transfusion microbiology. Vox Sang 64: 73-81, 1993
2. Barbara JAJ: Screening procedures for blood-borne viruses, in Harris JR (ed): Blood Separation and Plasma Fractionation, Wiley Liss Inc, 1991, pp 437-447
3. Zaaijer HL, Cuypers HTM, Reesink HW, Winkel IN, Gerken G, Lelie PN: Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341: 722-724, 1993
4. Linnen J, Wages J, Zhang-Keck Z-Y, Kirk EF, Krzysztos KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayinnis P, Fung K, Nakatsuji Y, Wia-Kuo Shih J, Young L, Piatak M, Hoover C, Fernandez J, Chen S, Zou JC, Morris T, Hyam KC, Ismay S, Lisson JD, Hess G, Foung SKH, Thomas H, Bradley D, Margolis H, Kim JP: Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 271: 505-508, 1996
5. Busch MP, Lee LLL, Satten GA, Henrard DR, Farzadegan H, Nelson KE, Read S, Dodd RY, Petersen LR: Time course of detection of viral and serologic markers preceding human immunodeficiency virus type 1 seroconversion: implications for screening of blood and tissue donors. Transfusion 35: 91-97, 1995

6. Zaaijer HL, Borg F, Cuypers HTM, Hermus MCAH, Lelie PN: Comparison of methods for detection of hepatitis B virus DNA. J Clin Microbiol 32: 2088-2091, 1994
7. McOmish F, Yap PL, Jordan A, Hart H, Cohen BJ, Simmonds P: Detection of parvovirus B19 in donated blood: a model system for screening by polymerase chain reaction. J Clin Microbiol 31: 323-328, 1993
8. Barbara JAJ: Hepatitis B variants: their importance in blood transfusion. Boletim 14: 148-9, 1992
9. Zuckerman MA, Hawkins AE, Barbara JAJ, Tedder RS: The identification of a hepatitis B transmission event by nucleotide sequence analysis. Transfus Med 4(Suppl 1): 52, 1994
10. Liang TJ, Bodenheimer HC, Yankee R, Brown NV, Chang K, Huang J, Wands JR: Presence of hepatitis B and C viral genomes in US blood donors as detected by polymerase chain reaction amplification. J Med Virol 42: 151-157, 1994