The polymerase chain reaction and hepatitis C virus diagnosis

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Abstract: In the absence of tissue culture, electron microscopy or assays for viral antigen, the direct detection of hepatitis C virus (HCV) is by necessity dependent upon nucleic acid hybridisation methods. Of the available methods, amplification of HCV cDNA by polymerase chain reaction (PCR) commends itself by virtue of its extreme sensitivity and its consequent ability to detect the very low levels of HCV-RNA that are present in many clinical samples. In this review the development and evolution of PCR techniques for HCV detection are described and a number of clinical applications are considered in detail. The applications include diagnosis of acute infection during the seronegative window period prior to the appearance of HCV antibodies, and diagnosis of HCV infection in the immunosuppressed. PCR also enables identification of the chronic viremic carrier state and it permits accurate monitoring of the antiviral effects of drugs such as interferon. Confirmation of the specificity of HCV antibody assays and detection of HCV contamination of blood donations and blood products are other important areas in which PCR techniques have proved invaluable. In addition, PCR-based techniques underlie an increasing number of molecular epidemiological and genotyping studies and they are providing insights into the details of HCV cellular tropism and replication. A number of logistic problems and operational difficulties are also discussed. Despite these limitations it is concluded that PCR will continue to make significant contributions to both clinical practice and to our understanding of the basic biology of HCV infection.

Key words: Polymerase chain reaction; Hepatitis C virus; Genome detection; Diagnosis; Quantification

Introduction

Since its introduction in the mid 1980s [1], the polymerase chain reaction has been employed for the detection of many classes of infectious agents including a wide range of viruses [2]. The technique has proved most useful for the identification of those viruses which are either completely non-cultivable or only cultivable with great difficulty. Latent infections and infections in which antigen production is limited or in which serological responses are delayed or absent are also particularly appropriate for diagnosis by PCR. The hepatitis C virus thus represents an ideal candidate for PCR-based detection because: (i) tissue culture methods for HCV isolation are not yet available; (ii) practical assays for HCV antigen have yet to be developed; (iii) identification of HCV in clinical specimens by electron microscopy is not practicable; and (iv) the antibody response to HCV is so delayed that diagnosis during the acute phase of the infection is fre-
Quently impossible. In this review the principles of PCR are outlined, and the development, applications, advantages and limitations of the technique in relation to hepatitis C are described.

**Principle of the polymerase chain reaction**

The PCR technique is capable of high level amplification of virtually any segment of DNA for which nucleotide sequence information is available. 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 twenty to several hundred base pairs (bp). A three-step temperature cycling process is performed: (i) denaturation of the double-stranded target DNA at high temperature (95°C) to separate the strands; (ii) reduction of the temperature (30–60°C) to allow annealing of the primers to the complementary sequence of each single-stranded DNA template molecule; (iii) temperature increase to 72°C to allow the thermostable enzyme Taq polymerase (from the bacterium *Thermus aquaticus*) to copy along both template 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 so that repeated cycling generates an exponential increase in the number of target DNA molecules (Fig. 1).

Amplification of approximately $10^7$ fold is obtained by 25–35 such cycles and even higher levels of amplification (up to $10^{12}$ fold) can be achieved by using 'nested' primers as described below. This extraordinary degree of sensitivity permits the detection of even single molecules (approximately $10^{-18}$ g) of the DNA sequence of interest. Although originally designed for DNA amplification, PCR can also be used to amplify RNA sequences if they are initially converted into complementary DNA (cDNA) by reverse transcription. Further details on general PCR principles, protocols and primer design are given in the methodology text of Innis and colleagues [3].

**Development of PCR techniques for HCV detection**

Since the initial description [4] of HCV detection by PCR, the assay has been adapted and improved by several groups. For purification of HCV-RNA from serum or liver tissue, proteinase K digestion [5], polyethylene glycol precipitation [6], silica absorption [7], acid guanidinium isothiocyanate-phenol-chloroform extraction [8] and a number of other methods have all been employed. Although most of these techniques are capable of producing viral RNA of adequate

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**Fig. 1. Polymerase chain reaction.** DNA is denatured at 95°C; oligonucleotides A and B are annealled, and extension from primers of a complementary DNA strand occurs in the presence of Taq polymerase and the four deoxyribonucleoside triphosphates (dNTPs). Subsequent cycles of denaturation, annealling and extension result in amplification of the segment defined by the 5' ends of the PCR primers. (Reproduced from Trends in Genetics (1989) 5(6); centrefold, with permission).
quality, we and others [9] have found the acid guanidinium protocol of Chomczynski and Sacchi [8] to be the method of choice at present. However, Van Doorn et al. [10] recently described a selective RNA-capture method which is both rapid and simple and has the advantage of eliminating the need for phenol-extraction and alcohol precipitation steps; the performance of this attractive alternative approach has yet to be fully evaluated.

Following RNA isolation all protocols include a cDNA synthesis step, primed either with specific antisense primer or alternatively with random hexamers. Both methods of priming are equally effective in most situations but random primed cDNA has the advantage of being amplifiable with PCR primers derived from any part of the genome. For convenience, and to minimise sample manipulations, some protocols are designed to permit cDNA synthesis and amplification in the same tube [11].

Both conventional single PCR [12,13] and double (‘nested’) PCR [5,11,14,15] protocols have been described. ‘Nested’ PCR, in which the products of amplification are reamplified using a second set of internal primers, has the advantage of increased specificity and the potential to detect single molecules of cDNA without the use of radioactive probes. The sensitivity of these assays is such that approximately 1 CID50 (1 CID50 = dose that infects 50% of chimpanzees) can be detected [16]. Amplification of HCV nucleic acid derived from formalin-fixed liver tissue is less efficient, however [17].

Oligonucleotide primer sets from several different parts of the HCV genome have been employed for PCR but many of these proved inadequate in the clinical context due to marked sequence heterogeneity between different HCV ‘isolates’ [16,18,19]. Detection problems arising from such sequence variation have now largely been overcome by the introduction of PCR primers based on the very highly conserved 5’ noncoding region of the genome [9,16,20,21]. Further improvements in the sensitivity of the assay have been achieved by the use of a primer set which amplifies a very short, approximately 60 bp, segment of the 5’ noncoding region [22].

Most of the ‘early’ publications on HCV-PCR described assays that were essentially qualitative. More recently, there have been reports of quantitative and semi-quantitative PCR assays, the development of which has been stimulated in part by the requirement for accurate monitoring of viraemia levels in relation to interferon therapy. Quantification methods based on limit dilution of cDNA [15,23,24] are effective but very labour intensive as are methods employing co-amplification of known amounts of competitive mutated templates [25,26]. Alternative, less labour intensive methods suitable for the analysis of large numbers of samples are currently being developed and evaluated [27,28].

Applications of PCR in HCV research and clinical management

**Diagnosis of acute HCV infection and of HCV carriage**

A major limitation of serological assays for antibodies to HCV (anti-HCV) is their inability to provide a diagnosis during the acute phase of the illness, due to delayed seroconversion [29]. We [6] and others [30,31] have demonstrated by means of PCR that there is frequently a period of anti-HCV seronegativity lasting from several weeks to several months during which HCV-RNA is the only marker of infection. Viraemia may even be detectable before the onset of hepatic dysfunction in some cases [31]. The implication of this finding for blood donor screening programmes is that assays for antibody alone are most unlikely to be able to eliminate all infectious donations from the blood supply. Unfortunately however, due to logistic limitations (see below), donor screening by PCR is not currently a feasible option.

Diagnostic problems with HCV-antibody assays not only arise during the seronegative window period at the onset of infection but also in patients who are unable to mount an immune response due to immunodeficiency or immunosuppression. The ability of PCR to circumvent this diagnostic difficulty has been demonstrated in both organ transplant recipients [32] and pa-
tients undergoing cytotoxic therapy for haematological malignancies [33].

In contrast to the transient HCV viraemia observed in cases of acute spontaneously resolving non-A, non-B hepatitis (NANBH), a viraemic carrier state lasting for many years is commonly seen in chronic NANBH [6,31]. Although, even in the presence of normal alanine aminotransferase (ALT) levels, persistent HCV viraemia is usually associated with histological evidence of hepatic damage [34], recent evidence suggests that this is not invariably so [35]. Intermittent viraemia patterns have also been described, both in humans and chimpanzees [6,31,36], but the significance and prognostic implications of such patterns have yet to be established.

A number of studies on HCV carriers have employed PCR to determine whether virus is present in body fluids other than blood. The findings have been somewhat inconsistent; Fried et al. [37] failed to detect HCV-RNA in either saliva or semen samples obtained from 12 carriers whereas Liou [38] detected HCV-RNA in both of these fluids. Other workers [39,40] have also found HCV genomes in saliva but not in breast milk. In addition, urine and ascites have been shown to contain HCV-RNA in a proportion of cases [38]. In view of the importance of establishing with certainty the mechanisms and routes of transmission of hepatitis C, further studies in this area are clearly warranted.

Viraemia quantification and monitoring of therapy

As mentioned above, various types of quantitative and semi-quantitative PCR assays for HCV-RNA are now available and these have been used to assess viral load in both untreated and interferon-treated patients. In untreated patients with chronic infection, most reports suggest a wide spread of HCV-RNA titres ranging from approximately $10^3$–$10^8$ genomes per ml of plasma [13,15,23], with levels up to 100 fold higher than this in the presence of immunosuppression ([33]; unpublished data). The extent of the relationship between the quantity of circulating HCV-RNA and the degree of hepatocellular damage is not known but a significant association between high HCV-RNA levels and high mean ALT concentrations in chronic NANBH has been reported [41].

Since the late 1980s many investigations have confirmed that interferon therapy is capable of producing an improvement in both histological and biochemical parameters of liver disease in a proportion of patients with NANBH [42]. Although the exact mechanism by which interferon reduces hepatic inflammation in this disease is unknown, several PCR-based studies suggest that the drug may have a direct suppressive effect upon HCV replication [23,43,44]. In a collaborative project with Brillanti and colleagues [23] we have shown a striking correlation between the clinical response to interferon and the decline in titre of circulating HCV-RNA. Furthermore, we [24] and others [44] have demonstrated that PCR is a sensitive and early indicator of the virological relapse that frequently occurs upon cessation of therapy (Fig. 2).

Taken together, these findings indicate that PCR will be an invaluable tool for monitoring the effect of antiviral drugs in NANBH. In addition, quantitative HCV PCR may also find a role in the selection of those most suitable for therapy, since pre-treatment HCV-RNA titre has recently been described as the strongest independent predictor of a sustained response to interferon [26].

Detection of HCV in blood donations and blood products

Following the introduction of blood donor screening for anti-HCV it soon became apparent that the relationship between serological reactivity in the antibody screening assays and infectivity of the donated blood was not a simple one. Prospective studies in both Amsterdam and London [5,45] showed that less than 20% of screen test reactive (anti-C100) donations were infectious. PCR for HCV-RNA has been used with success to differentiate between infectious and non-infectious donations and thus has helped minimise unnecessary loss of donors and facilitated donor counselling [5].

Studies of blood donations in the UK and elsewhere have demonstrated that positivity in supplementary serological tests, such as the second-generation recombinant immunoblot assay
(Ortho Diagnostics RIBA-2), correlates well with PCR positivity, and thus indirectly with both hepatitis C viraemia and infectivity [46]. Although this correlation will often eliminate the need for PCR analysis, it seems likely that PCR will still be required to aid interpretation of the many ‘indeterminate’ RIBA-2 results that arise in blood donor confirmatory work [47]. In this context it is noteworthy that one of the RIBA-2 reactivity patterns (5-1-1 plus c100-3 bands) regarded until now as definite evidence of HCV infection has inf~

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Fig. 2. HCV PCR monitoring of interferon therapy in a patient with chronic NANBH. Note the rapid ‘rebound’ in HCV viraemia that occurs within a month of stopping interferon therapy. (Figure adapted from Garson et al, [24], with permission).
of PCR testing of such concentrates before release may well contribute to increased levels of product safety in future.

Over the past few years there has been increasing use of intravenous immunoglobulin (IVIG) preparations to treat inherited agammaglobulinaemia and a number of other conditions. These preparations frequently contain antibodies to HCV at a level high enough to be detectable in recipients by anti-HCV immunoassays. The post-infusion anti-HCV seroconversion may give rise to anxiety over the possibility of HCV infection, although most studies indicate that the risk of this is extremely low [53]. In such circumstances PCR is the only method capable of differentiating between innocent passively acquired antibody and genuine HCV infection [54].

'Confirmation' of specificity of HCV antibody assays

In the blood donor screening context, PCR is commonly regarded as a 'confirmatory' test. The quotation marks are intended to point out that an antibody test result cannot strictly be confirmed by a test for viral nucleic acid. Although the presence of HCV-RNA in a blood donation strongly suggests that a positive anti-HCV test on the same sample represents a genuine antibody response, it must be remembered that this is only an assumption. Conversely, it is important to be aware that the absence of HCV-RNA in a donation does not necessarily prove that the associated antibody reactivity is false.

The problem of false-positive anti-HCV results has not only arisen in blood donor screening but also in investigating the potential role of HCV in chronic active hepatitis and in a number of other diseases including various autoimmune conditions [55,56]. Initial reports of HCV involvement in autoimmune hepatitis were doubted because of the potential confounding influence of hypergammaglobulinaemia and circulating immune complexes on the accuracy of anti-HCV tests. The controversy is not merely academic but has significant therapeutic implications because interferon, rather than corticosteroids, may be the rational approach to treatment if an aetiological role of HCV is confirmed. In collaboration with Bianchi and colleagues, we have been able to provide by means of PCR, independent non-serological evidence of HCV involvement in type 2 autoimmune hepatitis [57]. Similarly, PCR has been used to confirm the role of HCV in hepatocellular carcinoma [58] (Fig. 3), in mixed cryoglobulinaemia [59] and in porphyria cutanea tarda [60]. Involvement of HCV in idiopathic pulmonary fibrosis has also been suggested on the basis of anti-HCV testing [61], but this has not yet been confirmed by PCR analysis.

Molecular epidemiology and HCV genotyping

PCR amplification has become a powerful aid in the rapid generation of nucleotide sequence data. Thus, PCR-based studies are defining the extent of sequence variation and the mutation rates in different parts of the HCV genome [62,63]. The recognition that the amino terminus of the putative E2/NS1 protein contains a hypervariable domain has important implications with regard to vaccine design [64]; envelope hypervariability may also be a significant factor in the establishment of chronic infections through the generation of escape mutants.

PCR-based sequencing studies of variations among isolates are useful for both molecular epidemiological surveys of geographically diverse populations [65] and for defining routes and
mechanisms of transmission in local outbreaks and between individuals. For example, by sequencing PCR-generated envelope fragments, Innoue et al. [66] demonstrated mother-to-child transmission of HCV through two generations. Using a similar PCR approach, but based on core rather than envelope sequencing, Okamoto et al. [67] confirmed accidental transmission of HCV by needlestick injury.

Phylogenetic analysis of PCR-generated sequences from diverse isolates has revealed the existence of at least six distinct HCV genotypes [65,68,69]. Several different genotyping methods have been described, all are dependent on initial PCR amplification and involve either type-specific oligonucleotide primers [67], type-specific hybridisation probes [69] or type-specific restriction fragment length polymorphisms [70]. Such methods may become important clinically because a number of observations suggest that certain genotypes may be associated with more severe liver disease [70] and with either resistance to or response to interferon therapy [71]. Serological diagnosis based on antibody reactivity against recombinant HCV antigens may also prove to be genotype-dependent to some extent [70].

HCV replication studies and recombinant protein production

Very little is known about the replication and cellular tropism of HCV but, by analogy with flaviviruses and pestiviruses, HCV might be expected to be capable of infecting and replicating in mononuclear cells as well as in hepatocytes. This possibility has been explored in a number of studies by means of a PCR technique designed to be specific for the minus-strand (replicative intermediate). Some [72,73] but not all [74] of these studies have found evidence of HCV replication in peripheral blood mononuclear cells. The inconsistencies may be due in part to differences in the stringency of the controls employed to demonstrate strand-specificity. Minus-strand-specific PCR has also been used to provide supportive evidence for HCV replication in a human T-cell line [75]. Such techniques are likely to be utilised more frequently as many laboratories are now attempting to culture HCV in vitro.

In addition to the various clinical and basic science applications outlined above, PCR has been used widely as an adjunct to traditional cloning techniques in the production of the recombinant HCV proteins upon which most immunoassays are currently based. In the future, PCR may also prove to be a useful tool in the production of recombinant proteins for HCV vaccines.

Limitations of HCV-PCR

Despite the benefits and advantages of PCR in a wide range of HCV studies, several inherent limitations and logistic difficulties must be considered.

(i) The extreme sensitivity of PCR exacerbates the problem of false positivity due to contamination of the test sample. This is a particular problem in transfusion laboratories where automated sampling with washable probes is becoming increasingly common. In laboratories where such devices are in use, PCR will require separate sampling to avoid even minimal carry-over. Great care must also be taken to avoid contamination of samples by amplified PCR products from previous reactions. Sample preparation and amplification procedures should ideally be carried out in separate laboratories unless the uracil N-glycosylase (UNG) carry-over prevention system is used [76]. Strict application of the contamination avoidance measures outlined by Kwok and Higuchi [77] is essential. The importance of these measures has recently been dramatically illustrated by the findings of an international HCV-PCR quality control exercise in which 9 of 31 laboratories were shown to have generated false positive results [78].

(ii) If samples containing HCV are not rapidly frozen and stored under optimal conditions (at least −20°C) or if they are subjected to numerous freeze/thaw cycles, significant degradation of the labile RNA genome may occur. This may result in false negative PCR results [79].

(iii) PCR for HCV-RNA remains a relatively complex, time consuming and expensive assay. Typically, 20 samples take one person approximately 3 days to complete so that the assay as
currently performed is quite unsuitable for large scale applications such as blood donor screening. However, a rapid single tube protocol which allows cDNA synthesis and PCR amplification to be accomplished in under 90 min has recently been described [80]. Such a protocol in conjunction with microtitre plate format product analysis [27,28] may in future make PCR more applicable to large throughput applications.

**Conclusion**

The absence of tissue culture techniques, electron microscopic identification methods and assays for viral antigen made the direct detection of HCV in clinical samples and blood products a most challenging problem. Fortunately, PCR protocols for amplification of HCV cDNA were rapidly developed once sequence information became available, and the problem of HCV detection was thereby solved. However, the relative complexity of the technique, its high susceptibility to contamination and a number of other operational difficulties have largely limited its use to specialist research-orientated laboratories with expertise in molecular biology. In future this situation is likely to change as PCR becomes available to a much wider range of laboratories with the introduction of ‘user friendly’ HCV-PCR kits incorporating contamination prevention devices such as the UNG system. There is also a possibility that alternative non-PCR methods of HCV genome detection might be developed, although at present it is uncertain whether or not they will be able to match the extraordinary sensitivity of PCR [81,82]. Notwithstanding such developments, it seems likely that for several years at least, PCR will continue to make significant contributions to both clinical practice and to our understanding of the basic biology of HCV infection.

**References**


