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The polymerase chain reaction: essential for the development of curative therapy for hepatitis C

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Introduction

Hepatitis C virus (HCV), a single-stranded RNA virus, infects nearly 180 million people worldwide. The treatment of this chronic infection is currently undergoing one of the greatest ‘sea changes’ experienced in modern medicine in that a disease to which millions of deaths have been attributed globally could be virtually eliminated in our lifetime. This shift is due to the replacement of difficult-to-tolerate, injectable interferon-based therapies, given for up to a year with moderate chances of success, by all oral “direct acting antivirals” (DAA), specifically designed to act against HCV proteins including nonstructural (NS)3/4A (a serine protease), NS5A (a viral protein essential for viral assembly and RNA replication) and NS5B (a RNA dependent RNA polymerase). These new medications have markedly increased cure rates (over 90% in most cases), with far fewer adverse effects and shortened courses of therapy[1–3]. At the heart of this change to a more focused model of treatment has been the use of polymerase chain reaction (PCR)-based technologies (among others) in the understanding of key components of the HCV life-cycle and the design of DAAs. In conjunction with this, there has been on ongoing roll-out and expansion of PCR-based assays in regular clinical use for HCV.

Identification of HCV and development of DAA

At the time of the first reports describing PCR were published, HCV infection had been recognized for approximately 10 years as a separate clinical entity from hepatitis A and hepatitis B (termed non-A, non-B hepatitis or NANBH) [4, 5]. The use of interferon for treatment of NANBH was described even before the viral agent was identified (and named) in 1989 through screening of a cDNA library generated from NANBH serum[6, 7]. Progress from there was initially rapid, with the development of a serologic assay for diagnosis of HCV[8], PCR-based assays for the detection of HCV RNA in blood and liver tissue[9], the determination of the sequence of the HCV genome[10] and the identification of 6 main genotypes of HCV[11] all within a few years of identification. It was the knowledge of the sequence of the HCV genome and the use of cloning technologies that enabled

characterization of the RNA polymerase function of the NS5B protein [12, 13]. This knowledge, combined with the successful use of structural analysis and development of antivirals active against the HIV reverse transcriptase (another viral polymerase), contributed to the identification and development of nucleos(t)ide inhibitors (NI) and non-nucleoside inhibitors (NNI) of the HCV polymerase. Although it took many years, most interferon-free regimens currently in clinical practice such as sofosbuvir or dasabuvir contain NS5B inhibitors [1–3].

Despite this early flurry of activity in the early 1990s, further progress was hobbled by the lack of either a relevant animal or *in vitro* model for HCV infection. The only animal susceptible to HCV infection is the chimpanzee, which when infected does not suffer significant hepatitis or other liver disease. Nonetheless, this model did provide useful information – for example, RNA transcripts from cDNA clones of HCV sequences were infectious when directly inoculated into the liver of chimpanzees[14]. By selectively introducing deletions within in the HCV genome, the NS3/4A protease was identified as being essential for viral replication[15], and therefore as an attractive drug target[16]. Subsequently, the first DAAs developed for clinical use were the NS3/4A inhibitors telaprevir and boceprevir[17, 18], which have been largely replaced by the protease inhibitors simeprevir and paritaprevir, forming the basis of the available IFN-free DAA regimens [2, 19].

From a cell-culture standpoint it was only in 1999 that a subgenomic replicon was identified that could replicate in human hepatoma cells[20]. It was as late as 2005 when cell-culture based models which reproduced the entire viral life cycle and produced infectious HCV particles *in vitro* were initially reported [21, 22]. These cell culture systems enabled screening of potential antiviral compounds, identifying those that block viral entry or replication, monitored by measuring HCV viral load in culture by RT-PCR[23, 24]. Using a fully infectious culture model, NS5A was discovered as a potent antiviral target[25] indispensable for replication and assembly. This led in turn to the clinical development of the NS5A inhibitors daclatasvir and ledipasvir[1, 3].

PCR-based testing in routine clinical care

HCV viral load

Hepatitis C virus infection is diagnosed by testing for antibodies to HCV, an enzyme-linked immunosorbent assay (ELISA). These antibodies remain positive for life. Nevertheless, up to 25% of those infected with HCV will spontaneously clear their infection [26][27]. It is therefore essential for clinicians to be able to diagnose those with ongoing chronic HCV infection in order to identify those who will benefit from therapy.

As with hepatitis B surface antigen (HBsAg), it is possible to measure circulating HCV antigen[28]; however, quantitative HCV RNA viral load testing is essential to assess response to IFN-containing and DAA-based therapies. HCV viral load testing uses reverse transcription of HCV RNA to cDNA coupled with quantitative PCR based systems to accurately assess circulating HCV viral burden. Detection of HCV RNA in blood by these assays became possible very shortly after the identification of HCV[9], and was further

optimized once primers targeting more conserved regions of the viral genome were identified [29]. It became apparent that some patients who were infected with HCV (as determined by a positive HCV antibody) had actually cleared the virus (as determined by persistent absence of detectable HCV RNA in serum). Moreover, HCV RNA is detectable prior to the development of HCV antibodies, enabling earlier diagnosis of acute HCV infection, which has a superior response to IFN-based therapy compared to chronic HCV.

For those who began antiviral treatment, many “stopping rules” for IFN-based therapies required a sensitive quantitative assay; for example for simeprevir/IFN/RBV treatment, HCV RNA > 25 IU/ml at 4 weeks indicates treatment failure and that treatment should be discontinued. Interestingly, in the DAA era, these “stopping rules” are less applicable since detectable HCV RNA during DAA treatment is less predictive of the absence of virus several weeks after completion of antiviral treatment - the sustained viral response (SVR) - than it is for IFN-based therapies. Finally, arguably the most important use of HCV RNA assays is to determine the SVR[30], since a durable SVR, as defined by a negative or undetectable HCV PCR 12–24 weeks after completion of therapy, is unambiguously associated with a superior prognosis, associated with significant reductions of subsequent hepatic decompensation and mortality[31, 32].

HCV genotype

The 6 major genotypes of hepatitis C virus were recognized soon after the discovery of the virus through PCR amplification and sequencing with phylogenetic analysis[11]. This recognition was essential as different genotypes behave differently clinically and in their response to therapy[33]. In the IFN era (where all patients received IFN/RBV) HCV genotype determined the length of therapy and probability of SVR; however in the DAA-era, widely available PCR-based genotype identification (and in the case of genotype 1, subtype identification) is crucial as the activity of DAAs varies according to HCV genotype.

IL28B and interferon lambda 4

Until the development of DAA, the standard-of-care for treatment of HCV was once-weekly pegylated IFN combined with daily oral ribavirin, a difficult-to-tolerate treatment combination requiring 6–12 months of therapy associated with an SVR rate of ~ 40–50% for genotype 1 infection, significantly lower for African-American populations, compounded by difficulties predicting response to therapy. A genome-wide association study (GWAS) identified a single nucleotide polymorphism (SNP) near the *IL28B* gene, which was associated with a twofold difference in response to PEG-IFN- and ribavirin-based treatments[34]. Since those with *IL28B* CC genotype had significantly higher SVR rates than those with CT or TT genotypes, the low frequency of CC in African populations explained up to half of the difference in treatment response between this group and Caucasian populations. Further studies involving RNA sequencing identified a new gene and protein IFN lambda 4 (IFNL4), produced in association with the unfavorable TT genotype and associated with reduced response to therapy[35]. The development of commercial PCR-based *IL28B* genotype assays enabled practitioners to predict for their patients with reasonable accuracy the probability of responding to PEG-IFN-based therapy. The predictive value of this genotypic assay for DAA-based, IFN-free therapy appears to be

much lower, with most studies suggesting that *IL28B* genotype has no bearing on chance of achieving SVR [1–3].

Resistance testing

In the era of IFN-based immunomodulatory treatment for viral hepatitis, the issue of viral resistance was not given much consideration, since virally encoded IFN resistance of HCV is not described. Rather, the nonresponse to IFN may be attributable to impaired host antiviral responses. The development of DAA, which target specific viral proteins for treatment of chronic HCV, has changed the approach to resistance variants. Because of error-prone nature of the HCV polymerase, profound viral diversity and heterogeneity at all nucleotide sites occurs in the infected person. Since DAA exert selection pressure, viral variants, bearing mutations that confer resistance to these agents, can emerge, the detection of which can be tedious and expensive, requiring culture of the virus in the presence of drug (a so-called “phenotypic resistance assay”). PCR-based technologies enable rapid detection of specific mutations or “resistance associated variants (RAV)” associated with resistance to particular agents, such as the L31M mutation associated with a significantly reduced response to NS5A inhibitors. These mutations can then be specifically sought in patient samples without the use of cumbersome viral culture techniques (a “genotypic resistance assay”). These PCR-based assays are relatively cheap, with results available in ~ 1–2 weeks. With ultra-deep sequencing, RAVs are detectable in treatment-naïve individuals, although resistance testing pre-DAA therapy has yet to achieve clinical utility [36]. Nevertheless, while DAA such as sofosbuvir appear to have a high genetic barrier to resistance, the widespread use of antivirals (especially in difficult-to-treat populations) will likely increase the number of individuals harboring resistant viruses, requiring resistance testing for those who have previously failed DAA-based therapy.

Clinicians caring for HCV-infected patients will commonly order several routine PCR-based assays: quantitative HCV RNA testing, viral genotyping, and possibly the patient *IL28B* genotype. With widespread use of DAA-based therapy and a broader understanding of RAV, it is likely that genotypic HCV resistance assessment will become routine for those who fail DAA-based therapies.

It would have been difficult to imagine 30 years ago that PCR would become such an integral component of routine clinical practice. Although the development of DAA has been dependent on many technologies, without PCR and its impact on associated technologies such as sequencing and cloning, the understanding of the life cycle of HCV and the resultant development of the spectacularly successful directly acting HCV antivirals would not have been possible.

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