AMPLIFICATION OF HVR1 REGION OF HEPATITIS C VIRUS WITH PATIENT SPECIFIC NESTED PCR PRIMERS

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ABSTRACT

The understanding of the function of the hyper variable region (HVR) at the NH₂-terminus of the E2 protein remains very limited. Comparison of published sequences of HCV has led to the identification of a number of distinct virus types that may differ from each other by as much as 33% over the whole viral genome allowing the virus to escape cellular and humoral immunity. This phenomenon is related to the capacity of HCV to escape immune recognition, by frequent mutations HVR1 region. Several investigations have observed that the antibodies present in a proportion of infected patients had significant cross reactivity with apparently unrelated HVR1 sequences presented as synthetic peptides.

To study the HVR1 cross reactivity and evolution in individuals with varying liver disease severity, nested PCR was performed to obtain patient specific 150 bp long HVR1 genome. For this, previously amplified first round single molecule amplimers were reamplified using new patient specific sense and antisense primers. The patient specific sense primers did not work in the second round amplification. So, the target DNA was amplified by three rounds of nested PCR, using patient specific primers on the third round.

Key Words: *HCV, patient specific HVR1, nested PCR.*

INTRODUCTION

Hepatitis C virus

HCV is a positive strand RNA virus, and is a member of the Flaviridae.¹ HCV has been identified as the cause of post transfusion non A non B hepatitis.^{2,3} Different isolates of HCV show substantial nucleotide sequence variability distributed throughout the viral genome.⁴ Regions encoding the putative envelope proteins (E1 and E2 non- structural protein) are the most variable,^{5,6} whereas the non-coding region 5'NCR is the most conserved.^{7,8,9} The amino terminal end of E2 contains two hyper variable regions (HVR1 and HVR2) that exhibit significant variation amongst HCV isolates, over time within individuals. The variations of the nucleotides and amino acid sequences in the envelope proteins allow the virus to escape

the neutralizing antibodies.¹⁰ Comparison of published sequences of HCV has lead to the identification of a number of distinct virus types that may differ from each other by as much as 33% over the whole viral genome.^{1,4,11}

The degree of sequence variability is sufficient to alter the antigenic and biological property of the virus, so that the HCV infection may persist, because of inability of the host to mount a sufficiently efficient humoral and cellular immunity.¹³ This phenomenon is related to the capacity of HCV to escape immune recognition, by frequent mutations; particularly in the HVR1 region.^{14,15} Mutants capable of escaping from the neutralizing antibodies produced by the patients make immune therapy and vaccine development difficult. Ray et al 1999,¹⁶ hypothesized that the persistence may be directly related to

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Fig. 1 : Genomic organisation of HCV. The two ends contain untranslated regions (UTR)¹²

sequence variability in the envelope genes and immune response to HVR1. Sequence based studies have showed that the quasi species complexity and E1 dN/dS ratio (selective pressure, ratio of non synonymous to synonymous substitution) were lower in individuals with persistent viremia. In contrast, higher HVR1 dN/dS ratios were detected among those with persistent viremia.¹⁷ Studies on the rate of HVR (hyper variable region) evolution and complexity in hypogammaglobulinemic patients and in infants with delayed humoral immunity have showed reduced HVR variation.¹⁸

Thus in the absence of any selective pressures, new HVR variants remain minor species and the frequency of HVR genetic variation is apparently reduced. Thus assuming that E2 HVR variability is proportional to the HVR specific immune response and that viral persistence associates with increased HVR variability; this raises the question of whether antibodies to the HVR are protective or beneficial to the individual. Alternatively, HCV may use the HVR as an immune decoy an immuno-dominant region, to which immune response is non-protective.

The levels of variation and HVR complexity in different individuals during primary HCV infection highlight the contribution of both neutral evolution and selective forces in driving virus variability. Several investigations have observed that the antibodies present in a proportion of infected patients had significant cross reactivity with apparently unrelated HVR1 sequences presented as synthetic peptides.¹⁹ Establishment of a persistent infection occurs via different routes and probably in a typical 'Darwinian' fashion.

So, to study the HVR1 evolution in individuals with varying liver disease severity, nested PCR was performed to obtain patient specific HVR1 genome.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is an in vitro technique that allows the amplification of a specific DNA region lying between two regions of known DNA sequence. PCR is so

sensitive that, a single copy of gene can be amplified and extracted out of complex mixtures of genomic sequences and visualized as a distinct band on agarose gels. PCR can also be utilized for rapid screening for sequencing of inserts directly from aliquots of individual phage plaques or bacterial colonies.^{20,21}

The PCR amplifications are achieved by using oligonucleotide primers. The primers are extended on single -stranded denatured DNA (template)' by a DNA polymerase in the presence of dNTPs under suitable reaction conditions. This results in the synthesis of new DNA strands complementary to the template strands.

DNA molecule strand synthesis can be repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. Each repetition of strand synthesis comprises a cycle of amplification. Each new DNA strand synthesized becomes a template for any further cycle of amplification and / or the amplified target DNA sequences is selectively amplified cycle after cycle.

The first extension products results from the DNA synthesis on the original template and these do not have a distinct length, as the DNA polymerase will continue to synthesize new DNA until it either stops or is interrupted to synthesize the new DNA by the start of the next cycle. The second cycle extension products are also of indeterminate length; however at the third cycle fragments of target sequence are synthesized which are of defined length corresponding to the positions of the primers on the original template. From the fourth cycle onwards, the target sequence is amplified exponentially.

Thus, the final number of copies of the target sequence is expressed by the formula, $(2^n - 2n^0) \times$. (Where n = number of cycles, $2n$ = first product obtained after cycle 1 and second product obtained after cycle 2 with unidentified length, x = number of copies of the original template.)

Potentially after 20 cycles of PCR, there would be 2-2 fold amplification, assuming 100 % efficiency during each cycle. The efficiency of a PCR will vary from template to template and according to the degree of optimization that has been carried out.

Hot start PCR

The attribute of this method is the separation of one or more important components of the PCR such that all reaction components are mixed only after denaturation of the template. Originally this was achieved, by mixing the polymerase with a preheated reaction mixture. The benefits of hot start are particularly evident with amplifications from low target copy numbers because the reactants do not mix until the temperature is sufficiently high to melt the wax (55°-58° C). This minimizes any non-specific annealing of the primers to non-target DNA sequences and reduces the incidence of primer oligomerisation.²²

Nested PCR

Nested PCR primers are the ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second round PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. Using nested PCR method can dramatically increase the sensitivity and specificity of both DNA and RNA amplification. This technique eliminates any previous non-specific amplification products, because after the first round of PCR, any non- specific products are unlikely to be sufficiently complementary to the nested primers, to be able to serve as a template for further amplification. Thus the desired product, is preferentially amplified.²²

MATERIALS AND METHODS

The strategies for the study was as follows:

To amplify HVR1 region of E2 genome, previously amplified first round single molecule amplimers were reamplified using new HVR1 patient specific sense and antisense primers. Each primer pair was designed to take into account the HVR1 variability of each patient's quasi species within the primer site and to amplify a fragment of DNA encompassing only the

HVR1 region. (See fig 2 for primer map)

PCR protocol for the generation of HVR1 product

1. PCR set up with normal taq polymerase (Roche Diagnostics)

10 buffer - 2.5 ?l

dNTPs - 0.25 ?l

taq - 0.15 ?l

Water - 20.1 ?l

Primer 1 (sense) - 0.5 ?l

Primer 2 (antisense) - 0.5 ?l

24 ?l of the reaction mixture was pipetted in a clean RNase, DNase free thin walled PCR reaction tubes; two drops of mineral oil was added and then 1 ?l of the template DNA was added. For the negative control, water was added instead of the DNA template. The template was pipetted right into the reaction mixture, and was spun briefly. The PCR cycle (in the PCR machine, Peltier thermal cycler, PTC- 100 manufactured by MJ research, program) was 94°C / 45 sec, 50° C / 45 sec, 72° C / 90 sec, 34 times, 72° C for 10 min, 4° C forever (named as program E1E2B).

2. PCR reaction set up with hotstar taq

10 buffer - 5 ?l

dNTPs - 0.5 ?l

Sense primer - 1 ?l

Antisense primer - 1 ?l

Hotstar taq - 0.3 ?l

Water - 40.2 ?l

48 ?l of the mixture was dispensed in each tube and 2 drops of mineral oil added. 2?l of the sample template and 2 ?l water for negative control tube and PCR program, 94°C /10 min-hotstar step, followed by the above-mentioned program E1E2B. After the PCR reaction cycles were over, the products were visualised by running on the 2% agarose gel against low UV.

The first round, sense and antisense primers used, were E1 and E2 respectively and the second round sense and antisense primers were E3 and E4 respectively. For third round amplification, patient specific primers were used. (See fig. 2)

Fig. 2 : Primer map - location of the primers in the E2 region of HCV genome.

RESULTS

Previously, the entire E2 protein was amplified by nested PCR protocol to enable the expression and capture of the HVR1 peptides. The whole E2 genome was sequenced for single molecule amplimers by ABI automated sequencer. Major and minor variants at the HVR1 region were identified using appropriate sequence analysis software.

This previously amplified first round single molecule amplimers (using E1 and E2 primers) were reamplified using new patient specific sense and antisense primers, E3 and E4. Normal taq polymerase was used and PCR reaction was set up as described in the methodology. The first round primers E1 and E2 were located at about 800 and 1621 amino acid position in the entire E2 genome. So the size of the first round products was approximately 821. A negative control was set up, using water as the template (Sigma RNase Dnase free water) and a positive control was set up using E3 and E4 primers (previous experiments showed that these primers work for amplification of entire E2 genome). The PCR program was E1E2B.

The result was that the patient specific sense primers did not work in the second round amplification.

PCR optimisation

Different annealing temperatures were tried by hit and trial method finally the annealing temperature of 45°C seemed to work. But still we were not able to have convincing PCR products. So as an alternative, trouble-shooting protocols were followed using the different buffers available in the PCR optimisation kit (Invitrogen). About 16 different types of buffers were tried according to the PCR optimisation kit. This was also not successful.

So finally, there were no alternatives, but to change the strategy. It was postulated that the failure of the PCR was either due to the secondary structures present in the genome or the potential distance between E1/E2 primers and patient specific primers.

Third round PCR

Even though a third round PCR protocol is not a brilliant idea; it was performed using the second round products as template, as the PCR products for the HVR1 region was not amplified in the second round with patient specific primers.

So the experiment strategy was changed and second round PCR was set up using again E3 sense and E4 antisense primers that amplified the whole E2 genome. Then the second round product was diluted 1: 10, 1: 20, & 1: 40 by serial dilution with water. 1 µl of this serially diluted second round PCR product was used as a template to generate the third round

PCR products with the patient specific sense primers (at amino acid position aa 384) and E2BAS antisense primers (same as E4 but had BamHI restriction site incorporated; the primers contained restriction enzyme sites to allow directional in-frame cloning of the HVR1 PCR product. The PCR products were to be cloned in pre-cut pET-21 vector.) This spanned PCR product of 150 bp.

The negative control was set up using water as a template and positive control with E3 and E4 sense and antisense primers respectively.

The template was diluted so as to annul the carry over effect of the dNTPs, primers, and buffers of the second round reaction mixture.²³

When analyzed on 2% agarose gel, it showed that the HVR1 region (150 bp long approx.) was amplified in the third round with the patient specific primers. There was no carry over effect of the dNTPs and the primers; for this, negative controls were set up; one without primers, and another without dNTPs.

The bands on the gel were not bright enough, so that it could be used for cloning (for future experiment). Hence, as an alternative, instead of using normal taq polymerase, PCR was performed using hot star taq polymerase with double reaction volume of 48 µl. with the cycling program E1E2B with an initial hot star step of 94°C for 10 min was added. The amplified DNA showed comparatively bright band of 150 bp. (fig 3)

Fig. 3 : Amplification of HVR1 region with hotstar taq polymerase and patient specific primers (first and second lane; the third lane is 50 bp DNA ladder).

The result was such that the negative controls showed no carry over effects of primers, enzymes or dNTPs. There was no difference in the product concentration and primer dimer artefacts regardless of the template dilution. So, it was decided that 1: 40 dilution would be used as the template.

As the first round product was being used as the template, utmost care was taken while pipetting out the first round

product. It was made sure that the whole product was thawed properly if stored at -20°C & centrifuged briefly, so that the oil remained afloat and 1 μl of the template was pipette out carefully. At some point to minimise the errors in the pipetting the first round PCR products were diluted 5 times and 5 μl was loaded instead of 1 μl the calculating the volume of water to make up the final PCR reaction volume to be 25 μl .

DISCUSSIONS

Since the unveiling of the PCR method of DNA amplification at the American Society of Human Genetics Conference in Oct 1985, more than 600 publications involving the use of PCR have appeared in scientific literature. Numerous modifications, improvements and novel applications of PCR have been devised, yet there has been no source to which scientists could turn for basic instruction in the PCR method that is most suitable for the experimental problem at hand. Furthermore, there is no single set of protocols that work in every situation, even though some authors have drawn definitive conclusions from a single system about the importance or dispensability of certain parameters. Many extensions of the original PCR method have been published and numerous critical factors have been identified. Unquestionably, no single protocol will be appropriate to all situations. Consequently, each new PCR application is likely to require optimisation.²² Some often encountered problems include: no detectable product or a low yield of the desired product, the presence of non specific background bands due to mis-priming or mis-extension of the primers; the formation of 'primer-dimers' that compete for amplification with the desired product; and mutations or heterogeneity due to misincorporation.^{20, 22}

This study is an experimental protocol for PCR, using nested PCR in a special situation of amplifying hyper-variable region of HCV genome. At first the protocol was devised on one sample only. When it was successful, it was applied to all the other samples. There were altogether 18 samples. (7 major and 11 minor variants at the HVR1 region). The protocol was found to be applicable to all the 18 samples. Hence three round of nested PCR technique could be applied in other situations similar to this. The risk of contamination is a drawback of this extremely sensitive method.^{22,23}

CONCLUSION

As the second round nested PCR did not work with patient specific primers, we performed third round PCR to amplify 150 bp long HVR1 region of E2 genome of HCV with patient specific nested PCR primers.

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