CHAPTER IV

MATERIALS AND METHODS

PART I. Development of Nested RT-PCR for detection of HCV RNA

1. Chemical reagents and instruments

Most of the chemical agents used in this study were molecular biology grade and were purchased from Sigma, USA. Enzymes used in molecular technique were purchased from Promega, USA. Name lists of all chemical reagents and instruments were shown in appendices.

2. Oligonucleotide Primers

Oligonucleotide primers were synthesized with a DNA synthesizer (Gene Assembler Special, Pharmacia, LKB). Two sets of primers were generated from the 5' non coding region of the published sequences (105), which is highly conserved among HCV isolates. The external primers were sense, nucleotide positions -324 to -305, 5'-GGCGACACTCCACCATGAAT-3' (primer 1), and antisense, nucleotide position -17 to +3, 5'-CATGGTGCACGGTCTACGAG -3' (primer 2). The internal primers were as follow: sense, nucleotide position -291 to -271, 5'-GGAACTACTGTCTTCACGCAG -3' (primer 3) and antisense, nucleotide position -52 to -32, 5'-TCGCAAGCACCCTATCAGGCA -3' (primer 4). The position of these two sets of primers on hepatitis C virus genome was depicted in figure 4.

3. Extraction of nucleic acid

In order to obtain more efficient and easier nucleic acid extraction, two different procedures were set up and compared. A serial of ten fold dilution of positive

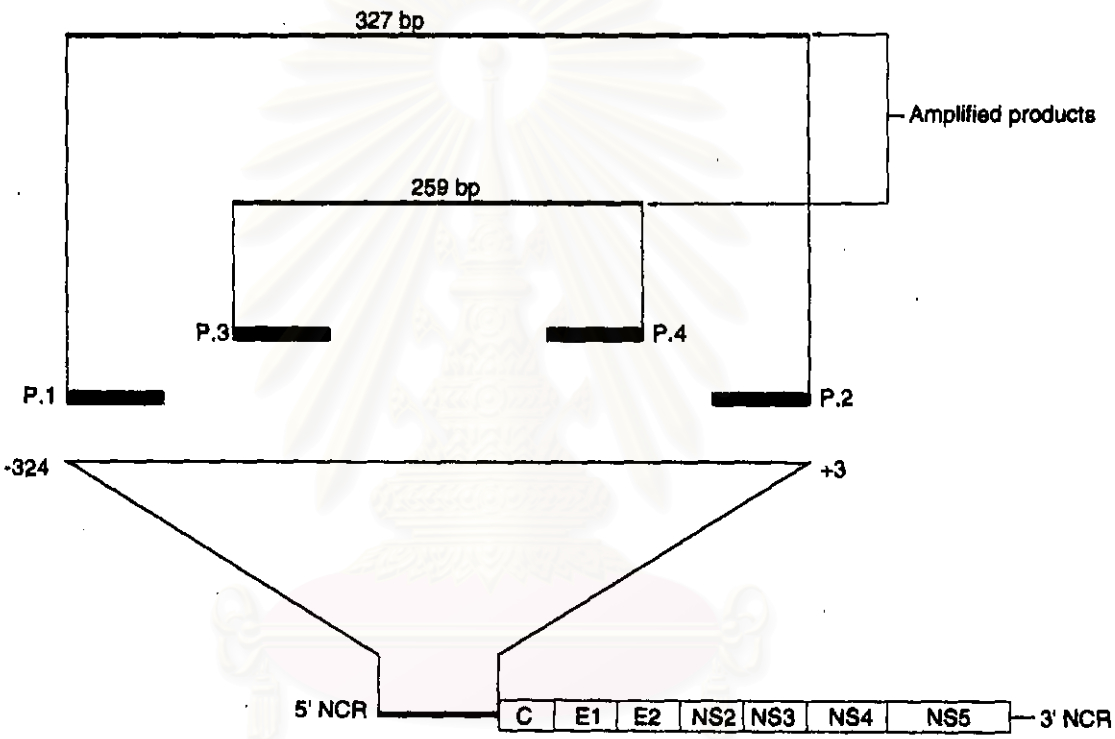

Figure 4. The positions of the primers for PCR localized on Hepatitis C virus genome. P1 = primer 1, P2 = primer 2, P3 = primer 3 and P4 = primer 4.

control plasma sample which contain approximately 8×10^7 genome eq/ml according to quantitation in bDNA (Quantiplex HCV RNA, Chiron) was used to compare these procedures.

3.1 Nucleic acid extraction was prepared by Proteinase-K-sodium dodecyl sulfate (Proteinase-K/SDS) method (90) with the following modifications. To a 1.5 ml eppendorf tube, 200 ul of serum were added to an equal volume of buffer containing 0.2 mol/l 'Tris'-HCl pH 7.5, 25 mmol/l EDTA, 0.3 mol/l NaCl, 2% (w/v) SDS and 200 ug/ml proteinase-K. The mixture was thoroughly mixed and incubated at 37^0 c for 1 hour. The protein was extracted twice by adding 400 ul of phenol-chloroform-isoamylalcohol (25:24:1) into the mixture and mixed extensively on the vortex mixer at maximum speed for 3 minutes. Then, it was centrifuged at 14,000 rpm for 10 minutes at room temperature. The aqueous phase was seperated out of the organic phase which was at the bottom. The inner phase was mainly protein. The aqueous phase was transfered without disturbing the interface to a fresh eppendorf tube, containing 400 ul phenol-chloroform-isoamylalcohol, and the extraction was repeated once more. The residual trace of phenol was eliminated by extraction with 400 ul of chloroform-isoamylalcohol (24:1). The aqueous phase was transfered to a new eppendorf tube, followed by adding 40 ul of 3 M Na acetate and 800 ml of cold absolute ethanol. The solution was mixed and RNA was precipitated at -70^0 c for 30 minutes. RNA was pelleted by centrifugation at 14,000 rpm for 15 minutes at 4^0 c and the supernatant was discarded. The pellet was washed once with 1 ml of cold 70% ethanol. After centrifugation and supernatant discarded, the pellet was vacuum dried for 10 minutes. The final RNA was dissolved in 40 ul of DEPC-treated water and stored at -70^0 c, or used immediately.

3.2 Nucleic acid extraction was prepared by a single guanidinium isothiocyanate (GuSCN) treatment method (106). Six hundred microliters of lysis solution containing 5.75 M GuSCN, 50 mM 'Tris'-HCl pH 7.5, 100 mM β-mercaptoethanol, and 1 ug of poly (rA) per ml was taken into a 1.5 ml eppendorf tube,

followed by adding 150 ul of serum sample. The resulting lysate was mixed and incubated at 65^0 c for 10 minutes. After incubating, RNA was precipitated by adding 700 ul of isopropanol at room temperature into the lysates. Then, it was centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded. The RNA pellet was washed once with 1 ml of 70% ethanol. The RNA pellet was recovered again by centrifugation at 14,000 rpm for 10 minutes at room temperature and was vacuum dried for about 10 minutes. Finally, the RNA was dissolved in 30 ul DEPC-treated water and stored at -70^0 c.

4. Amplification of HCV RNA by Nested RT-PCR.

4.1 Reverse Transcriptase (RT) step.

cDNA synthesis was carried out in a total volume of 28 ul in a 0.5 microtube. Ten ul of RNA template was added to 18 ul of RT reaction mixture containing 5 ul of 5x RT buffer (250 mM 'Tris'-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 4 ul of 25 mM $MgCl_2$, 6 ul of 2.5 mM dNTP, 1 ul of 50 pmol/ul primer 2, 0.5 ul of 40 U/ul RNasin, 0.5 ul of 200 U/ul M-MLV reverse transcriptase, and 1 ul of DEPC-treated water. This mixture was covered with 2 drops of mineral oil. Reverse transcription was performed in a Gene Amp 480 thermocycle (Perkin- Elmer Cetus) with one cycle at 37^0 c for 30 minutes, and then at 99^0 c for 5 minutes.

4.2 First PCR amplification.

The first round of PCR amplification was performed in the cDNA reaction mixture. A total volume of 22 ul PCR mixture containing 2.5 ul of 10x buffer for Taq polymerase (50 mM KCl, 100 mM 'Tris'-HCl pH 9.0 at 25^0 c, and 1% Triton x-100), 1ul of 50 pmol/ul primer 1, 0.5 ul of 5 U/ul Taq polymerase and 18 ul of DEPC-treated water was added to each cDNA tube under the oil layer. The thermocycle was programmed to denature samples at 94^0 c for 3 minutes and to amplify through 40 cycles with each cycle consisting of 94^0 c for 1 minute (denaturation), 55^0 c for 1

minute (primer annealing), 72^0 c for 1 minute (extension) and a final extension step at 72^0 c for 5 minutes.

4.3 Nested PCR.

The nested PCR was carried out under mineral oil in a 48 ul of reaction mixture containing 5 ul of 10x buffer, 4 ul of 25 mM $MgCl_2$, 4 ul of 2.5 mM dNTP, 1 ul of each 50 pmol/ul primer 3 and primer 4, 0.5 ul of 5 U/ul Taq polymerase, and 32.5 ul of DEPC-treated water. Two microliters of the first PCR product were transferred to the reaction mixture. Nested PCR was performed for 35 cycles in the thermocycle, programmed as for the first PCR amplification.

5. Detection of amplification products.

Ten microliters of nested PCR product were analyzed by electrophoresis on a 1.5% agarose gel, consisted of 50 ug/ml Ethidium bromide, in a Tris-borate EDTA buffer (PH 8.0). The electrophoresis was carried out at 150 volts for 30 minutes. The PCR product was visualized under UV and a photography was taken by a handheld polaroid camera.

6. Quality control in each run of Nested RT-PCR.

Negative control plasma sample was a qualified plasma unit donated from a healthy repeated donor without risk factors of viral hepatitis. This plasma tested negative for HB_sAg, HIV-Ag, anti-HIV, anti-HCV and HCV-RNA. Positive control plasma sample was an anti-HCV and HCV-RNA positive sample that was known to contain HCV RNA approximately 8×10^7 geq/ml. The positive control plasma dilution 10^{-4} and 10^{-5} (contained about 8×10^3 and 8×10^2 geq/ml, respectively) were used, in order to determine run to run sensitivity.

In each experimental run, a negative control and two positive control plasma (dilution 10^{-4} and 10^{-5}) were carried through all of the steps in the procedure from extraction to product detection. To avoid false positive with PCR, strict application of

the contamination prevention measures of Kwok and Higuchi was followed (107). For a run to be considered valid, the negative control had to test negative and both dilutions of positive control had to test positive.

Part II. Determination of the sensitivity of Nested RT-PCR technique.

The PELICHECK HCV RNA sensitivity panel was used to evaluate sensitivity of the procedure. PELICHECK HCV RNA contains two dilution panels of HCV genotype 1 and genotype 3. This sensitivity panel has been included in the second EUROHEP collaborative study. After exclusion of laboratories that generated erroneous results on the panel, 44 high quality laboratories were selected. These 44 laboratories produced 56 data sets of sufficient quality. The results on the PELICHECK dilution of the EUROHEP genotype 1 and 3 plasma standards were presented in table 1 and table 2, respectively. For adequate sensitivity the dilution 1: 4000 of genotype 1 and 3 plasma standards should be reactive to reach the 50% participants PCR dilution endpoint.

The genotype1 and 3 plasma standards were extracted by Proteinase-K and Guanidinium isothiocyanate, followed by Nested RT-PCR as above.

Part III. Detection of HCV RNA in blood donations.

1. Study group.

All specimens investigated in the study were obtained from routine blood donations at NBC, TRCS, between January 1994 and December 1994. Everyday about 50 blood donor sera were analyzed for ALT level and also routinely screened for HB_sAg (Abbott 3 rd generation EIA), HIV Ag (Abbott, EIA detect P24 Ag), Anti-HIV (Abbott 3 rd generation EIA) and Anti-HCV (Abbott 2 nd generation). Sera from donors who were positive for HB_sAg, HIV Ag, and anti-HIV were not included in this study. Study samples were divided into 4 groups according to anti-

HCV status and ALT level. Group 1 comprised 100 blood donations negative for anti-HCV with normal serum ALT level ($\leq$ 56 IU/ml), group 2 composed of 314 blood donations negative for anti-HCV with elevated serum ALT level (> 56 IU/ml), group 3 were made up of 89 blood donations positive for anti-HCV with normal serum ALT level, and group 4 comprised 79 blood donations positive for anti-HCV with elevated serum ALT level.

2. Specimen collection.

Blood donor samples were collected into a sterile tube as an extra sample from routine donations. The selected blood samples were seperated by centrifugation at 2500 g for 10 minutes within 6 hours of collection. The sera were aliquoted into two tubes, 1 ml volume, and frozen at -70^0 c until being thawed for analysis.

3. Anti-HCV screening assay.

Anti-HCV screening was performed on the COMMANDER system with Abbott HCV EIA-2 nd generation (ELISA-2). The ELISA-2 was an enzyme-linked immunoassay which detected antibodies against the recombinant antigens C100-3, C33c (nonstructural antigen), and C22-3 (structural antigen, core). Tests were performed according to the manufacture's instructions. The cut off value was mean absorbance of the negative control plus 0.25 times the mean absorbance of the positive control, which was automatically calculated by the COMMANDER. Sample with absorbance values equal to or greater than the cut off value were interpreted as reactive. Initially reactive samples were retested in duplicated and were considered ELISA positive if either duplicate retested was reactive. Samples with absorbance values less than 10 percent of the cut off values were considered negative.

4. ALT Testing.

ALT values were determined by a kinetic method using an automatic analyzer (CCX, Abbott Spectrum System, USA). The upper limit of normal was 56 IU/ml, defined according to the manufacturer's instructions.

5. Statistical analysis.

The chi-square test (X^2) was used to compare the differences among groups. A p value ≤ 0.01 was considered significant.

Table 1. HCV-RNA detection rates by different PCR methods in the PELICHECK dilution series of the EUROHEP genotype 1 plasma standards as reported in 56 data sets of 44 qualified laboratories.

Eurohep panel number	dilution	genome eq./ml[1]	Nested PCR		Single PCR		Roche Amplicor		Total	
			n.	%	n	%	n	%	n	%
EU-HCV25	100	36,000	33	100	7	100	16	100	56	100
EU-HCV09	1000	3,600	26	79	5	71	14	88	45	80
EU-HCV11	4000	900	18	55	5	71	12	75	35	63
EU-HCV15	16000	225	12	36	0	0	2	13	14	25
EU-HCV17	64000	56	5	15	0	0	1	6	6	11
EU-HCV19	256000	16	4	12	0	0	0	0	4	7
EU-HCV21	1024000	4	1	3	0	0	0	0	1	2
EU-HCV23	4096000	1	0	0	0	0	0	0	0	0

Table 2. HCV-RNA detection rates by different PCR methods in the PELICHECK dilution series of the EUROHEP genotype 3 plasma standards as reported in 56 data sets of 44 qualified laboratories.

Eurohep panel number	dilution	genome eq./ml	Nested PCR		Single PCR		Roche Amplicor		Total	
			n.	%	n	%	n	%	n	%
EU-HCV24	10	120,000	33	100	7	100	16	100	56	100
EU-HCV22	100	12,000	32	97	7	100	14	88	53	95
EU-HCV20	1000	1,200	23	70	5	71	9	56	37	66
EU-HCV18	4000	300	15	46	1	14	5	31	21	38
EU-HCV16	16000	75	6	18	0	0	1	6	7	13
EU-HCV14	64000	19	2	6	0	0	0	0	2	4
EU-HCV10	256000	5	0	0	0	0	0	0	0	0
EU-HCV26	1024000	1	0	0	0	0	0	0	0	0