CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Population study

To validate the in-house 5' non coding region (NCR) direct DNA sequencing assay, twenty six specimens submitted for HCV genotyping to the Molecular Microbiology Laboratory, Ramathibodi Hospital, Bangkok, Thailand were used. All specimens were previously identified HCV genotypes by using TRUGENE HCV 5' NC genotyping kit (Visible genetics, Canada).

One hundred HCV infection submitted for HCV genotyping to the Molecular Immunology Laboratory, King Chulalongkorn Memorial Hospital, Bangkok, Thailand from October 2001 through December 2003 were studied. All specimens were positive for HCV RNA using the in-house Polymerase Chain Reaction (PCR). The in-house PCR generates a 264 base pair target from the 5' NCR which used for genotyping analysis.

1.2 Collecting specimens

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Plasma specimens were collected by standard procedure with ethylene diaminetetraacetic acid (EDTA) as the anticoagulant. Serum specimens were removed from the clot within 6 hours of blood collection by using centrifuge at 1,500 rpm 10 min. All serum and plasma specimens were frozen and store at –20 ^{0}C or –70 ^{0}C until use.

2. Methods

2.1 HCV RNA Extraction

Serum or plasma specimens were used for extraction of HCV RNA by using QIAamp® Viral RNA Extraction Mini kit (QIAGEN, Germany). The extraction procedure was modified from QIAamp® Viral RNA Mini kit handbook. Five hundred and sixty microliters of prepared Buffer AVL containing Carrier RNA was pipette into a 1.5-ml microcentrifuge tube. One hundred and forty microliters of serum or plasma was added to the buffer AVL. Then the solution was mixed by pulse vortexing for 15 s. The mixture was incubated at room temperature (15-25 ^{0}C) for 10 min. After briefly centrifugation, the five hundred and sixty microliters of ethanol were added to the sample and mixed by pluse vortexing for 15 s.

After briefly centrifugation, the six hundred and thirty microliters of mixture was removed to the spin column (in a 2-ml collection tube) and centrifuged at 8,000 rpm for 1 min. The spin column was placed into a clean 2-ml collection tube and discarded the tube containing the filtrate. This step was repeated. Five hundred microlitters of Buffer AW1 were added and centrifuged at 8,000 rpm for 1 min. The buffer AW2 were added into spin column and centrifuged at 14,000 rpm for 3 min. The spin column was removed to 1.5-ml microcentrifuge tube and fifty microlitters of buffer AVE was added and incubated at room temperature for 1 min, then centrifuged at 8,000 rpm for 1 min. The HCV RNA extract was frozen and stored at –20°C until used.

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2.2 RT-PCR amplification

The primers are consist of

Primer 1: nucleotides –325 to -305; 5'- ACACTCC(G/A)CCATG(G/A)ATCACT-3'

Primer 2: nucleotides –13 to 7; 5'-TGCTCATG(T/G)TGCACGGTCTA-3'

Primer 3: nucleotides –296 to -276; 5' GGAACTACTGTCTTCACGCAG-3'

Primer 4: nucleotides –52 to -32; 5' TCGCAAGCACCCTATCAGGCA-3'

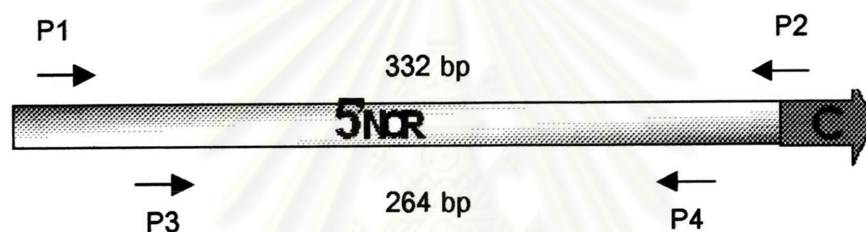

Figure 4. The position of primers performed an in house 5' NCR PCR.

RT-PCR was performed using the primer with complementary sequence located in the 5' NCR that are conserved among known HCV genotypes. RT-PCR was performed in the applied Biosystems GeneAmp 2400 PCR instrument. For RT-PCR reaction, 10 ul of extracted nucleic acid was combined with 20 ul of RT-PCR master mixture. RT-PCR master mixture contained 5X MML-V RT buffer, 25 mM $MgCl_2$, 1.25 mM dNTPs, 40 U of Rnase inhibitor, 200 U of Reverse transcriptase and 50 pmol reverse primer2 and Diethypyrocarbonate treated distilled water (DEPC H_2O). Reverse transcription was performed at 37°C for 30 min, followed by 99°C for 5 min for inactivation of RT.

For first PCR amplification, 20 ul of PCR master mixture; contained 10X PCR buffer, forward primer1, 2.5 U of Taq DNA polymerase and DEPC H_2O was added. PCR amplification consisted of preincubation step at 94°C for 5 min. After that, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s was performed and following by a final extension at 72°C for 5 min.

The nested PCR reaction was performed using two primers consisted of the forward primer 3 and the reverse primer 4. Each 48 ul nested PCR reaction contained 10X PCR buffer, 25 mM $MgCl_2$, 50 pmol of each primers, DEPC H_2O and 2.5 U of Taq DNA polymerase. Two microliters of PCR product was added into the nested PCR master mix. After the preincubation step at 94°C for 5 min, nested PCR amplification was 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s following by a final extension at 72°C for 5 min.

The negative control sample was a DEPC H_2O. Positive control sample was an anti-HCV and HCV RNA positive sample. In each experimental run, a negative and positive control samples were used in the procedure from amplification to product detection. For an experiment considered valid, the negative control had to test negative and the positive control had to test positive.

To prevent carryover contamination within the laboratory, all purchased reagents were split into small aliquots. The preparation of PCR reagents, the extraction of nucleic acids from clinical samples, and the amplification step were conducted in three different rooms. Tips equipped with scaling filters were used for pipetting of the reagents introduced into the PCR. All area and equipment were decontaminated with sodium hypochlorite prior to and after pipetting (78).

2.3 Agarose gel electrophoresis

To detect the PCR product, agarose gel of 2% was prepared by completely dissolving the gel powder upon heating in 1X TAE (89 mM Tris-HCL pH 7.4, 89 mM acetic acid, 2.5 mM EDTA). The solution was boiled in microwave oven until it was completely dissolved. When the solution temperature was around 50°C, it was poured into an electrophoresis chamber set with comb. Ten microliters of nested PCR product were mixed with Loading Dye and loaded into gel slots in submarine condition with 1.0 ul ethidium bromide (10 mg/mL). Electrophoresis was performed at 100 volts for 40 min. The positive band was visualized under UV light at 302 nm. The size of product was approximately 332 base pair in length for first round RT-PCR and 264 base pair for nested RT-PCR product.

2.4 PCR product purification

QIAquick PCR purification kit (QIAGEN, Germany) was used for purification of PCR products from amplification reaction. Forty microliters of nested PCR products were added to 200 ul of binding buffer and placed into a spin column. After centrifuged at 10,000 rpm for 1 min, the DNAs were adsorbed with silica-gel membrane. The unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides were removed. The pellet was washed with 750 ul of washing buffer and eluted the DNA with 50 ul of elution buffer. The concentration of PCR product was determined by measuring the absorbance at 260 nm

2.5 Sequencing

HCV genotyping of all HCV RNA positive specimens was attempted by direct sequencing of the amplification products generated in the detection assay in the Molecular Immunology Laboratory, King Chulalongkorn Memorial Hospital. The sequencing were performed by using BigDye™ Terminator v 3.1 Cycle Sequencing Ready Reaction kit

(Applied Biosystems, Foster City, CA). The concentration of HCV cDNA in mastermix was 40-60 ng/ul. The concentration of PCR template was calculated by

Conc. Of PCR product = O.D. value X dilution factor X conversion factor (50)

The sequencing reaction containing 2.0 ul of terminator ready reaction mix, 1.0 ul of 3.2 pmol sequencing primer, calculated template and H_2O were added until the final volume of reaction was 10 ul. The 25 cycles with following temperature cycling parameters; 95 °C for 10 s of denaturation, 50 °C for 5 s of annealing and 4 min of 60 °C for extension.

The extension product was precipitated by Ethanol/Sodium Acetate precipitation in 1.5 ml centrifuge tube. The precipitate solution was performed by the following reagents; 3.0 ul of 3 M sodium acetate (pH 4.6), 62.5 ul of 95% ethanol and DW for 14.5 ul. The 20 ul of reaction mixture was added to precipitation solution and incubated at room temperature for 15 min to precipitate the extension product. After centrifuged at 16,400 rpm for 20 min, the pellet was washed with 250 ul of 70% ethanol and vacuum centrifuge dried for 10 min. The extension product pellet was resuspended in 10 ul of Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer. The BigDye Terminator v 3.1 (dRhodamine) Cycle Sequencing reaction was analyzed by capillary electrophoresis (ABI 310 Genetic Analyzer, Applied Biosystems, Foster City, CA). The sequence was analyzed by Sequencing analysis Software and Sequence navigator Software before use in Genotyping analysis.

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2.6 Phylogenetic tree

Sequences of the in-house 5' NCR of HCV cDNA from those specimens were typing by the Clustal W Software, version 1.4. The following sequences from http://hcv.lanl.gov/content/hcv-db/index (79) were included in the analysis as reference sequences of 5' NCR in each genotypes and subtypes.

Table 3. The reference sequences of HCV 5' NCR in each genotypes.

Genotype	Reference sequences of each genotype	Total
1a	1a.FR.HPCSTRUCTA, 1a.GB.HCVRNACEE, 1a.JP.HC-J1, 1a.US.HCV-H, 1a.US.HCV-PT	5
1b	1b.AU.HCV-A, 1b.CN.HEBEI, 1b.DE.HCV-AD78, 1b.JP.HCV-J, 1b.JP.HCV-N	5
2a	2a.G2AK1, 2a.K-0041, 2a.NDM59, 2a.JP.HC-J5, 2a.JP.Td-6	5
2b	2b.JPUT971017, 2b.MD2B-1, 2b.QC5, 2b.JP.HC-J7, 2b.JP.HC-J8	5
2c	2c.BEBE1	1
2e	2e.ID.JK020, 2e.ID.JK109, 2e.ID.JK128	3
2f	2f.ID.JK081, 2f.ID.JK139	2
2k	2k.MD.VAT96	1
3a	3a.CB, 3a.NZL1, 3a.QC10, 3a.QC8, 3a.FR.HPCSTRUCTC	5
3b	3b.JP.HCV-Tr, 3b.TH.TH527, 3b.TH.TH576	3
3k	3k.ID.JK030, 3k.ID.JK049, 3k.ID.JK055, 3k.ID.JK070	4
4a	4a.QC11, 4a.QC12, 4a.QC13, 4a.JP.HEMA51	4
5a	5a.QC14	1
6 variants	6a.VN.VN506, 6a.VN.VN538, 6a.VN.VN569, 6a.VN.VN571, 6b.TH.TH580, 6d.VN.VN540, 6d.VN.VN787, 6d.VN.VN843, 6d.VN.VN998, 6f.TH.TH976, 6g.ID.JK046, 6g.ID.JK065, 6g.ID.JK148, 6h.VN.VN004, 6h.VN.VN085, 6i.TH.TH555, 6i.TH.TH602, 6j.TH.TH553, 6k.VN.VN405, 6k.VN.VN507, 6k.VN.VN530, 6k.VN.VN531	22

1.6 Data calculation

Prevalence of each HCV genotype in population

$$= \frac{\text{Number of each HCV genotype}}{\text{All of patients in population}} \times 100$$

1.7 Alignment of HCV sequence in each genotype in population

To identify other difference position in each genotype, the first PCR products of each genotype which is 332 bps in length were sequenced. The sequences were aligned with their references by Clustal X program.

The extended positions of each genotype were compared with previously positions reported by Stuyver, et al (72). All of these positions are located within 170 bp of 5' NCR. There are

R1 = nucleotides -240 to -233

R2 = nucleotides -167 to -155

R3 = nucleotides -147 to -142

R4 = nucleotides -138 to -132

R5 = nucleotides -128 to -118

R6 = nucleotides -100 to -92

R7 = nucleotides -81 to -70

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