CHAPTER V

RESULTS

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

The amplified products of the first and nested round of the PCR using these two sets of primers (primer 1 and primer 2, primer 3 and primer 4) would give rise to the amplified product of 327 bp and 259 bp respectively, as predicted from the prototype hepatitis C virus sequence (figure 4 and figure 8).

In this study, the efficacy of two nucleic acid extraction procedures, the Proteinase-K/SDS method and a single step GuSCN treatment method have been compared. They were evaluated on a serial of ten-fold dilution of the positive HCV RNA control sample. The amount of HCV RNA genome in the diluted samples were ranged from 8 $x10^7$ to 80 geq/ml. RNA template of each dilution was used to transcript to cDNA at 37^0 c for 30 minutes, followed by 99^0 c for 5 minutes. The first PCR amplification was performed in cDNA tube by adding the first PCR reaction mixture into cDNA tube and the mixture was denatured at 94^0 c for 3 minutes, then amplified through 40 cycles of 94^0 c for 1 min, 55^0 c for 1 min, 72^0 c for 1 min, and followed by 72^0 c for 5 minutes for complete extension. Two ul of the first PCR product were transferred to the nested PCR reaction mixture. The mixture was denatured and amplified for 35 cycles as the same programmes used in the first PCR amplification. Ten ul of nested PCR product were applied on an agarose gel for electrophoresis running. The resulting amplified product was visualized and photographed during UV light exposure. Figure 5 showed that nucleic acid extraction with Proteinase-K/SDS resulted in a 2 log higher detection of HCV RNA than that extraction with GuSCN method. Using Proteinase-K extraction, HCV RNA can be

detected from undiluted serum to dilution 10^{-5}, whereas using GuSCN extraction, HCV RNA can be detected from undiluted to 10^{-3} of positive control sample.

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

The PELICHECK HCV RNA containing two dilution panels of HCV RNA genotype 1 and 3 were used to evaluate the sensitivity of Nested RT-PCR technique. The dilution panel of genotype 1 contain 36,000, 3,600, 900, 225, 56, 16, 4 and 1 geq/ml. The dilution panel of genotype 3 contain 120,000, 12,000, 1,200, 300, 75, 19, 5 and 1geq/ml. The results showed that up to 1:4000 dilution (containing 900 geq/ml) of genotype 1 (figure 6) and up to 1:4000 dilution (containing 300 geq/ml) of genotype 3 (figure 7) plasma standard could be amplified when extraction with Proteinase-K method. Then, the sensitivity of Nested RT-PCR assay as performed was about 9 $x10^2$ and 3 $x10^2$ HCV RNA copies/ml, or 45 and 15 HCV RNA copies per assay for genotype 1 and 3, respectively. The nucleic acid extraction with GuSCN resulted in detection of HCV RNA at maximum dilution 1:100 of both genotype 1 and 3 which contained 36 x 10^3 and 12 $x10^2$ geq/ml, respectively. Therefore, extraction with Proteinase-K yielded better sensitivity for detection of HCV RNA than that extraction with GuSCN.

The successful development of this sensitive Nested RT-PCR (Proteinase-K extraction method) was used to detect the HCV RNA in blood donations in this study.

Part III. Detection of HCV RNA in blood donations.

Between January and December 1994, after exclusion of donations with positive for HB_sAg, HIV Ag, and anti-HIV, a total of 10,699 donations were determined for ALT levels. Of these, 10531 donations were anti-HCV seronegative, whereas 168 (1.6%) donations were anti-HCV seropositive. Of the 10,531 anti-

HCV seronegative donations, 9,535 (90.5%) had normal ALT level, 873 (8.3%) had midrange ALT elevation (57-112 IU/ml) and 123 (1.2%) had a high ALT elevation (>112 IU/ml). 314 samples (191 of midrange , 123 of high ALT elevation) (Group2) which represented elevated ALT samples derived from 3,220 voluntary blood donations and 100 samples with ALT normal (Group1) which were used as control, were selected to detect for the presence of HCV RNA. All of the 168 anti-HCV seropositive samples, 89 with normal ALT level (Group3) and 79 with elevated ALT (43 had midrange, 36 had high ALT) (Group4) were detected for the presence of HCV RNA. The clinical and biochemical characteristic of the blood donors were shown in table 3.

Results of anti-HCV seronegative samples were shown in table 4. None of 314 samples with elevated ALT was HCV RNA positive, likewise none of the 100 samples with normal ALT were viremic.

Results of anti-HCV seropositive samples were shown in table 5. One hundred and five (62.5%) of the 168 anti-HCV seropositive samples were HCV RNA positive. Table 6 showed the distribution of ALT level and the HCV RNA positive rate in relation to ALT levels. HCV RNA was detected in 35 of 36 (92.7%) in sera with high ALT level, 36 of 43 (83.7%) in sera with midrange ALT level, and 40 of 89 (38.2%) in sera with normal ALT level. Therefore, HCV RNA was more frequently detected in anti-HCV seropositive with elevated ALT than those with normal ALT ($X^2 = 49.09$, $p < 0.01$).

Table 7 showed the distribution of ELISA OD value and HCV RNA positive rate in relation to ELISA OD value. Samples were grouped according to OD values in ELISA assay. In group of OD > 2.0, 89 of 100 (89%) samples were HCV RNA positive. Samples with OD > 1.0-2.0, 9 of 22 (40.9%) were HCV RNA positive. Finally, in group with OD = cutoff-1.0, only 7 of 46 (15.2%) samples were positive

for HCV RNA. Therefore, HCV RNA was significantly detected in higher OD group than lower OD group ($X^2 = 78.21$, $p < 0.01$).

Positive rate of HCV RNA in relations to ELISA OD and ALT level in the 168 anti-HCV seropositive donations were summarized in table 7. The results indicated that blood donations with ELISA OD lower than 1.0 with normal serum ALT level, only 5% of them were HCV RNA positive. On the other hand, HCV RNA was detected in all but one donations showing ALT value higher than 112 IU/ml, irrespective of ELISA OD value.

In this study, if considering the detection of HCV RNA by PCR as gold standard, the sensitivity of anti-HCV antibody assay (Abbott EIA2) was 100%. Since of the 105 HCV RNA positive donations, all were positive with anti-HCV by ELISA-2 (Table 8). Among the 477 HCV RNA negative donations, 414 were negative with anti-HCV. Therefore, the specificity of ELISA-2, as calculated in PCR negative samples was 86.8%. Use of the ELISA assay to predict viremia was also evaluated in this study. The positive predictive value of the ELISA result was 62.5% and the negative predictive value was 100%.

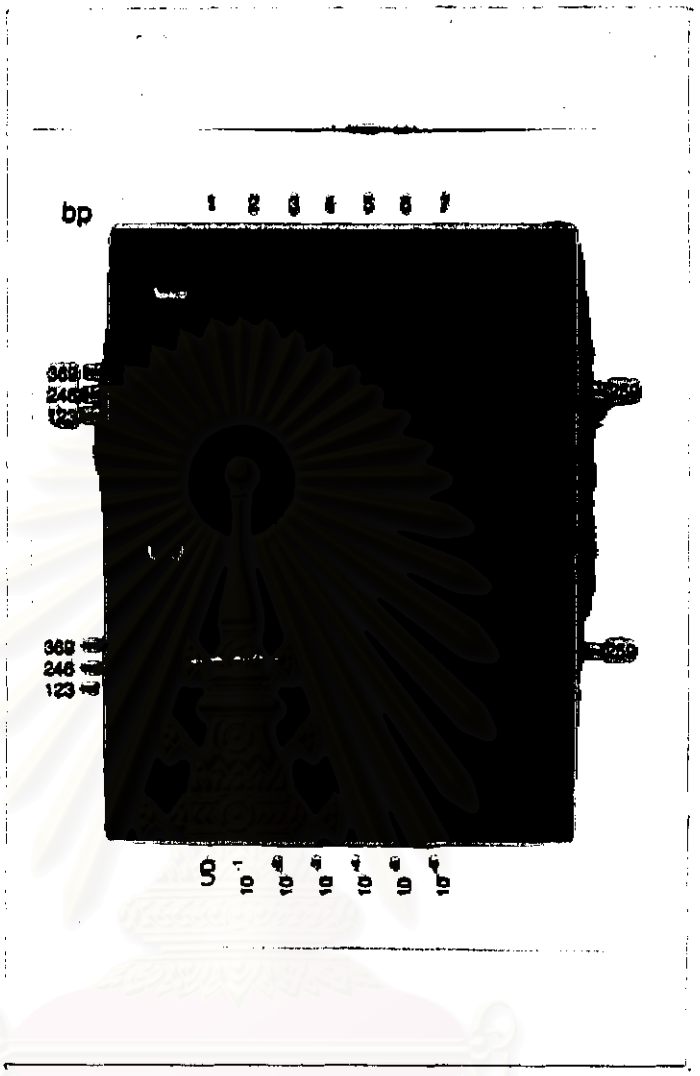

Figure 5. Agarose-gel electrophoresis showing amplifications of the serial dilutions of positive control sample which were prepared by two different methods. The upper part, the amplified products were derived from nucleic acid extraction with Guinidinium isothiocyanate method. The lower part, the amplified products were derived from nucleic acid extraction with Proteinase-K/SDS. Lane 1 was the molecular size marker of 123 bp. Lane 2 to 7 were amplified HCV RNA obtained from the amplifications of ten-fold serial dilutions of the HCV RNA positive control sample. Sizes of DNA standards and second-round of PCR product were designated in base pairs (bp)

Figure 6. Agarose-gel electrophoresis showing amplifications of the PELICHECK HCV RNA sensitivity panel of genotype 1 plasma standard. Lane 1 was the molecular marker of 100 bp. Lane 2 to 9 were amplified HCV RNA obtained from the amplifications of dilution panel of HCV RNA genotype 1 plasma standard. Lane 10 was a negative control plasma sample.

Figure 7. Agarose-gel electrophoresis showing amplifications of the PELICHECK HCV RNA sensitivity panel of genotype 3 plasma standard. Lane 1 was the molecular size marker of 100 bp. Lane 2 to 9 were amplified HCV RNA obtained from the amplification of dilution panel of HCV RNA genotype 3 plasma standard. Lane 10 was a negative control plasma sample.

Figure 8. Agarose-gel electrophoresis of amplified HCV RNA obtained from nested-PCR amplifications of seven serum samples which were positive for anti-HCV antibodies. Lane 1 was the molecular size marker of 100 bp. Lane 2 was negative control plasma sample. Lane 3 to 9 were anti-HCV positive samples after PCR amplifications. Lane 3 to 6, and 9 were positive but lanes 7 and 8 were negative for HCV RNA. Lane 10 and 11 were the positive control plasma sample dilution 10^{-4} and 10^{-5}, respectively.

Table 3. Clinical characteristics of blood donors which were divided into four groups according to anti-HCV status and serum ALT levels.

Characteristics	Group 1	Group 2	Group 3	Group 4
No. of donors	100	314	89	79
Anti-HCV status	-ve	-ve	+ ve	+ ve
ALT (IU/ml)	≤ 56	> 56	≤ 56	> 56
range	8.0 - 55.3	61.0 - 405.0	8.0 - 55.4	57.0 - 443.7
mean ± SD.	24.8 ± 11.5	115.2 ± 49.6	26.7 ± 13.1	130.7 ± 80.1
Sex (M/F)	76 / 24	276 / 38	72 / 17	73 / 6
Age (Yr)				
range	19 - 61	18 - 65	17 - 48	19 - 55
mean ± SD.	36.2 ± 9.9	39.1 ± 9.4	27.6 ± 8.1	28.7 ± 8.2

Table 4 . Detection rate of HCV RNA in anti-HCV seronegative blood donations: correlation to ALT level.

ALT level (IU/ml)	n	Negative HCV RNA (%)	Positive HCV RNA (%)
> 112	123	123 (100)	0
57 - 112	191	191(100)	0
≤ 56	100	100(100)	0
Total	414	414(100)	0

Table 5. Positive rate of HCV RNA in anti-HCV positive blood donations and correlation to ALT level.

ALT level (IU/ml)	n	Positive HCV RNA (%)
> 112	36	35 (97.2)
57 - 112	43	36 (83.7)
≤ 56	89	34 (38.2)
Total	168	105 (62.5)

Table 6. Positive rate of HCV RNA in anti-HCV positive blood donations and correlation to ELISA OD value .

Anti-HCV ELISA OD value	n	Positive HCV RNA (%)
> 2.0	100	89 (89)
> 1.0 - 2.0	22	9 (40.9)
cut off - 1.0	46	7 (15.2)
Total	168	105 (62.5)

Table 7. Positive rate of HCV RNA in anti-HCV positive blood donations: correlation with ELISA OD value and sera ALT level.

ALT IU/ml	Anti - HCV (ELISA-2) optical density											
	> 2.0			>1.0 - 2.0			cut off - 1.0			Total		
	n	+ve HCV RNA	%	n	+ve HCV RNA	%	n	+ve HCV RNA	%	n	+ve HCV RNA	%
> 112	31	31	(100)	2	1	(50)	3	3	(100)	36	35	(97)
>56-112	34	30	(88)	5	4	(80)	4	2	(50)	43	36	(84)
≤ 56	35	28	(80)	15	4	(27)	39	2	(5)	89	34	(38)
Total	100	89	(89)	2	9	(41)	46	7	(15)	168	105	(63)

Table 8. Correlation between the detection rate of HCV RNA by PCR and anti-HCV antibody by ELISA among 582 blood donations.

Anti- HCV test (ELISA- 2)	HCV RNA by Nested RT-PCR		Total
	Positive	Negative	
Positive	105	63	168
Negative	0	414	414
Total	105	477	582