CHAPTER VI

DISCUSSION

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

A Nested RT-PCR was developed in this study for detection of serum HCV RNA by modification method of Garson et al (90). A single step GuSCN treatment method (106) was applied to extract HCV nucleic acid in order to obtain easier extraction procedure. We compared the efficacy of this GuSCN extraction with a conventional Proteinase-K/SDS extraction procedure for the detection of HCV RNA in serial ten-fold dilution of positive control plasma sample and in PELICHECK HCV RNA sensitivity panel. Although nucleic acid extraction with Proteinase-K/SDS was more labour intensive, it yielded 2 log higher analytical sensitivity, when the quantity was assessed in a RT-PCR titration assay. Studies from Ulrich et al (108) indicated that the purity of template RNA was important since non nucleic acid components of plasma or serum, which can inhibit the cDNA-PCR reaction may contaminate the extraction. It was probably that in single step GuSCN extraction, the RNA template has not been purified, some inhibitory components from plasma/serum may also be co-extracted and influence the outcome of the HCV-cDNA PCR.

Recently, studies on reliability of PCR for detection of HCV RNA (93) were concluded that one-third of the laboratories had errors in determining the status of undiluted samples, and one-half had one or more errors in the dilution series. Only 5/31 (16%) of participating laboratories generated no errors, and even in these 5 laboratories reported a 100-fold difference in sensitivity for the dilution series. False negative results were largely due to lack of sensitivity whereas false positive results were commonly due to contamination. Our studies using Proteinase-K/SDS extraction

followed by Nested RT-PCR amplification, we estimated that the detection limit of this assay was approximately $3x10^2$ to $9x10^2$ geq/ml, which corresponded to 15 to 45 geq per assay. This level of sensitivity have met the requirement of the PELICHECK HCV RNA sensitivity panel for HCV RNA detection. The detection limit per assay of our PCR was also comparable to those reported by other investigators that HCV RNA was detected in 10-100 copies per assay (92,109). Moreover, in each experimental run, the positive control sample dilution 10^{-4} and 10^{-5} (containing $8x10^3$ and $8x10^2$ geq/ml) were included and carried from extraction through the nested PCR assay to ensure run to run sensitivity. Furthermore, we used primers in the 5' non-coding region of the HCV genome, which was the most conserved sequence (>92%) among the different viral isolates (83) in order to yield a good sensitivity of the PCR. Since false positive results were a major problem in PCR, in addition to employing the most stringent precaution (107), a negative control plasma was also subjected to all of the step in each experimental run to ensure that contamination did not occur. Thus, the Proteinase-K/SDS extraction followed by PCR amplification developed in this study was sensititive and reliable for the detection of HCV RNA in blood donors.

Detection of HCV RNA in blood donations.

To reduce the risk of transfusion associated NANBH/HCV, before the identification of HCV, donors were screened for surrogate markers such as ALT and anti-HBc. Data from several studies have established that ALT screening of blood donors prevented approximately 30-60% of post-transfusion HCV infections (110,111,112). Screening of blood donors with the first generation anti-HCV assay have further reduced post-transfusion HCV by 80-90% (68,113). The recent introduction of the second generation ELISA for HCV antibody have reduced PTH to levels that are indistinguishable from the background incidence of hepatitis in non-transfused hospitalized patients in USA (41).

A wide variety of factors unrelated to infectious disease such as age between 25 and 35 years, male gender, obesity, and the use of alcohol have been found associated with elevated ALT (114). Therefore, ALT screening has both low sensitivity and low specificity in identify HCV infection than a specific anti-HCV assay. However, ALT screening has been found useful for detecting hepatitis C infection during the window period (10,11,115,116). Moreover, studies by Sugitani et al reported that about 30% of HCV viremic donors with elevated ALT may be negative by second-generation anti-HCV tests (117). In our studies of anti-HCV seronegative donations, we were not able to detect any HCV RNA in all donations with high ALT level. We were also unable to detect any HCV infection from anti-HCV seronegative with midrange ALT level. Although our sample size was not large enough for firm conclusions to be drawn, the data suggested that serum ALT elevation did not reveal any HCV infection in approximately 3,220 volunteer blood donations in the study. The results in our study also confirmed the finding of other studies (109,118,119,120). This indicated that ALT elevation in seronegative blood donors was generally due to factors other than HCV infection, at least in our donor population. Data from our study showed that the donations with high ALT level accounted for 1.2% of total donations, in which these blood units were discarded and resulted in permanent deferral of the donors. All together, it was indicated that ALT screening of blood donors may lead to an unnecessary loss of safe blood and the chance of engendering needless anxiety among blood donors rejected solely because of elevated ALT. Therefore, our study suggested that ALT screening in addition to anti-HCV screening on every unit of blood donated may not be necessary. These have already been suggested by some countries (110,118).

In this study, using the second generation anti-HCV ELISA by Abbott, we detected an overall 1.6% prevalence of anti-HCV in blood donor population, a rate similar to that previously reported (121). The prevalence of HCV RNA in blood donors who were positive for anti-HCV antibody was 62.5%. This percentage was higher than those reported from Scotland (21.2%) (122) and France (25.9%) (123), but

was comparable to those reported from Spain (65%) (124) and Italy (56%) (120). Taken together, these results confirmed the suggestions of Sakugawa et al that HCV RNA positive rate among anti-HCV positive blood donors was higher in prevalence areas of anti-HCV than in lower prevalence areas (125). Among the anti-HCV positive donors, HCV RNA was detected in 97.2% of high ALT level and 83.7% of midrange ALT level, in contrast only 38.2% of normal ALT level (Table 6). Thus, it was obvious from our study that a good correlation was observed between the detection rate of HCV RNA and the ALT level in anti-HCV positive donors. This result was in agreement with a number of studies indicating that donors who were anti-HCV positive which elevated ALT had high prevalence of HCV viremia, ranging from 80-95% (120,126,127,128). By contrast, those anti-HCV positive donors with normal ALT level showed low prevalence of HCV viremia ranging from 15-42% (120,126,127,89,10).

Our study also demonstrated that anti-HCV positive donors, those with higher ELISA OD value had a significantly increase rate of detecting HCV RNA compared to those with lower ELISA OD value. This result was in agreement with other previously reported (10,89,129,130). Some also showed that high positive ELISA OD values were very likely to be true positive values, while a number of low positive ELISA OD values represented false positive results (89,129), based on the RIBA and HCV RNA testings. It was shown from our results that HCV RNA was detected in 89% and 40.9% in group with ELISA OD > 2.0 and >1.0-2.0, but only 15.2% in group with ELISA OD = cut off-1.0 (Table 7). Whether the latter group represented some false positive ELISA assay could be revealed by following these donors for sometime.

The HCV RNA was detected in all but one case showing ALT values higher than 112 IU/ml, irrespective of ELISA OD (Table 8). This was suggested that nearly all cases with ALT higher than double normal upper limit values could be diagnosed as HCV carriers, only if they were found to be positive by second generation anti-

HCV assay (Abbott), at least in our study. It was also shown that the higher OD value > 2.0 in ELISA-2 was a useful indicator of potential HCV infectivity, since 89% of them was HCV RNA positive and was diagnosed as HCV carriers. On the contrary, the donors of ELISA OD lower than 1.0 with normal ALT values only 5% was HCV RNA positive which suggested that more than 95% of them might not be HCV carriers. It was noteworthy that the question of whether anti-HCV positive but HCV RNA negative samples were due to resolve of infection, low grade viremia, or even failure of the PCR assay could be answered only if these donors have been followed up for sometime. Recent studies suggested that donors who were anti-HCV positive with persistently normal ALT values, but HCV RNA negative (on repeated samples) may probably have recovered from hepatitis C infection (95,128). Guninian-Paraz et al, however, have reported that a number of blood donations positive with weak ELISA OD value in ELISA-2 (Abbott) were found negative with other three "third generation" ELISAs and also negative for HCV RNA by PCR, which were indicated as false positive results from the assay (89). The anti-HCV positive but HCV RNA negative in our study especially those of ELISA OD lower than 1.0 with normal ALT remained unclear whether they had low-titered antibody as a memory of past infection with HCV or the tests result were non-specific. Additional studies, including donors follow-up, would be needed to definitively resolve these types of reactivities.

Considering the PCR test as gold standard, the second generation anti-HCV ELISA of Abbott showed a sensitivity of 100% and specificity of 86.8% in this study. Although the positive predictive value was low, the ELISA-2 assay was appropriate as a screening test for preventing transfusion-transmitted HCV infection. The direct detection of HCV RNA could be useful as a confirmatory test to identify viremic carriers. This would also help to minimize unnecessary loss of donors and to facilitate donor counseling.