CHAPTER VI

DISCUSSION

Currently, blood donor screening for hepatitis B virus is HBV surface antigen (HBsAg), for hepatitis C virus is anti-HCV. Although the risk for transfusion-transmitted diseases infection is extremely low, there is still a small chance that blood donated by infected individuals before seroconversion can escape the blood safety-screening procedures. For HCV, the preseroconversion window period is currently estimated to be 82 days based on second-generation antibody assays (range, 54 to 192 days)(75,76) and 70 days for – more recently developed third-generation antibody assay(13,77). Nucleic acid amplification technology could significant reduce the window period by an estimated 59 days relative to current third-generation antibody assay, resulting in an infectious window period of approximately 11 days (78). For HBV, there was some reports indicated HBsAg negative in blood donors but HBV DNA positive (3,4,5) and documented about HBsAg mutation, which may possible failure of commercial assays in detecting HBsAg (6). Detection of viral nucleic acids by PCR might enhance the safety of blood supply during the window period that would have been missed by conventional screening testes.

Several methods have been developed to simplify HCV RNA PCR, to optimize its sensitivity and specificity, and to reduce its disadvantages (78,79). Nested HCV RNA PCR techniques have been reported that circumvent the need to open the reaction mixture for addition of the second set of oligoprimers and, thus minimize the risk of contamination. In contrast, others have optimized one- stage methods to achieve sensitivities comparable with those obtained with the nested technique (80). One-step HCV RNA PCR methods have also been developed that combine both RT and PCR amplification steps (81). The one-step methods not only reduce the risk of contamination, but also reduce labor-intensity, time required for analysis, and cost. In addition, minimizing the number of steps could facilitate standardization and reduce variability in the HCV RNA PCR results among different laboratories.

In this study, the detection of both HBV DNA and HCV RNA by multiplex PCR was performed using DNA and RNA that has been extracted from pooled donated blood by Boom Extraction method; the NucliSens Basic Kit (Organon Teknika, Durham, NC) (74). The advantages of one-step HCV RNA PCR that combines RT and PCR amplification in a single tube containing both reverse transcriptase and *Taq* DNA polymerase, include elimination of addition of reagents between the RT and PCR steps, simplifies the procedures, minimizes the potential contamination (81). Base on the principles of one step PCR, a multiplex PCR technique was envisioned to detect simultaneously HBV DNA and HCV RNA. In our study, oligonucleotide primers of HCV: 5′ non-coding region was chosen by the most conserved sequence (>92%) among the different viral isolates (72) in order to yield a good sensitivity of PCR. The oligonucleotide primers of HBV was chosen from pre-core and core region which highly conserved among the HBV isolated; subtype adr, the most epidemic subtype in Thailand.

The sensitivity of the multiplex PCR for the detection of HBV DNA and HCV RNA was assessed in serum containing a wide range of known concentrations of HBV DNA and HCV RNA. The detection limits (Fig. 13 and Fig. 14) were 100% approximately 19,629 copies/ml (392 copies/assay) for HBV DNA and 100% approximately 4,506 copies/ml (90 copies/assay) for HCV RNA. Hu, K.Q. et al (81) reported the comparable sensitivities of combined HBV/HCV PCR and the conventional HBV DNA PCR or one-step HCV RNA PCR. It was 100% concordance between the results. In the study of Hu, K.Q. et al, it had the difference from this study in oligonucleotide primers of HBV and HCV and method of DNA and RNA extraction. Boom method (74) was used in this study. But phenol/chloroform extraction was used in Hu's study. It was performed in acidic environment (pH 4.0) to isolate RNA and adjusted pH 8.0 to isolate HBV DNA.

The boom method is base on the binding of nucleic acid in the presence of a chaotropic agent (quanidium thiocyanate (GuSCN) onto silica particles. Purification of nucleic acid is achieved by washing the silica with several solvents where by bound/free separation is done in conseccutive steps. After washing, the silica particles are dried, and the nucleic acid is eluted from the particles. The detection limit of combined NucliSens™and Amplicor test for HCV was determined at 40-50 geq/ml, based on Eurohep and VQC strand dilution series (Lelie et al.). The Food and drug Administration (FDA) is recommened for HCV NAT, a minimum sensitivity level

of 5,000 copies per ml at individual donation level (82). In this study, the sensitivity for HCV RNA in pooled sample was approximately 4,506 copies per ml.

For blood donor screening, PCR technology, especially multiplex HBV DNA/HCV RNA PCR, may have implications for future testing protocols since it is able to reduce in HBV and HCV window periods. PCR technology does not allow the screening of individual blood donations, because of the high cost-effectiveness, work over load, so pooling of samples would decrease the cost of PCR-base screening. The characteristics of multiplex HBV DNA/HCV RNA must be validated and evaluated before its introduction into pool testing for: reliability and reproducibility, sample storage requirements, optimal sample handing requirements, reduction in window period, sensitivity and specificity.

There have been reported for non-nested PCR internal control (18-20). Li, X. et al. was constructed the nested primer PCR of proviral HIV-1 DNA (21). The internal control template for a competitive nested primer PCR of HBV was constructed in this study. This construct shares the primer recognition sequences with the wild type template and yields a 200 bp fragment after amplification (the product of wild-type was 266 bp) to provide an internal control for the individual PCR runs. This nested primer control was introduced into a reaction tube containing the specific sample. The reason for qualitative the amount of internal control is to avoid being competitive with small of target in case internal control more than target. If the target is much more than internal control, the internal control cannot be seen as internal control is competed PCR reaction. Under the PCR conditions used, it found that 25 copies of the nested primer control reproducibly gave a positive PCR signal and did not contain components capable of interfering with the PCR process. Thus, the constructed control template is a proper internal control to allows excluding of false negative in nested primer PCR of HBV DNA.