CHAPTER V

DISCUSSION

The efficiency of the PCR technique is influenced by different parameters , the most important one seems to be the composition of primers, both in relation to each other and in relation to the target DNA, and the temperature at which annealing of primers is performed (31). Our primers were selected from the DNA sequence of common plasmid of *C. trachomatis* by "OLIGO" program (150) which also selected the annealing temperature of the reaction. The plasmid was found in all serovars of *C. trachomatis* and has no known homology with other organisms (142). In addition , there are 10 copies of the plasmid in one EB of *C. trachomatis*. It provides higher copy number of target DNA than other sequences and thus increases the sensitivity of the PCR.

By using our designed primers (primers A and B), the amplified product analyzed by electrophoresed in 1.5% agarose gel is a single band of 621- bp fragment without non-specific band or primer-dimer formation. The concentration of Mg^{2+} is important. It is required for the reaction of *Taq* DNA polymerase. The concentration of about 1.5 mM is usually optimal with 200 uM each dNTP, but in some circumstances, different amounts of Mg^{2+} may prove to be necessary. Generally, excess Mg^{2+} results in the accumulation of non - specific amplification products and

insufficient Mg^{2+} reduces the yield (151).

The concentration of *Taq* polymerase is an important parameter. Using excess amount of enzyme may result in greater production of non-specific PCR products and reduce yield of the desired target fragment (151). Accordingly , we determined the least enzyme concentration that gives satisfactory yield of the desired product. The optimal *Taq* polymerase in our PCR was 2.0 unit per 50 ul of reaction.

After the PCR reaction mixture was set, the optimal number of cycles was determined by varying the cycle at 30, 35, 40, 45, and 50 cycles. When the cycle was repeated 30 times, the PCR provided satisfactory yield of the desired amplification products. Slight increase in the yield after 40 cycles was obtained and remained unchanged after that. That is, after a certain number of cycles, the desired amplification product gradually stops accumulating exponentially and enters a linear or stationary phase. The plateau effects is due to competition between the formation of the specific primer template complex and the reannealing of the PCR product before finding a primer. In addition, as the amount of product increases, *Taq* DNA polymerase may become limited because there is less than one molecule of *Taq* per primer-template complex.

the experiment shows that 30 cycles is sufficient for obtaining satisfactory yield of the products with less time consuming.

False positive due to amplicon carryover was prevented by the use of dUTP instead of dTTP and uracil DNA glycosylase (UDG) in the reaction. By incubating the reaction at 37°C for 10 min, the UDG removes uracil residues from single stranded and double stranded DNA. Increasing the temperature to 94°C for 10 min, the UDG was inactivated and phosphodiester bond in abasic site of DNA was hydrolysed result in the breakdown amplicons of into small fragments. As deoxynucleotide triphosphates appear to quantitatively bind Mg^{2+} , the amount of dNTPs present in a reaction will determine the amount of free Magnesium available. When we used dUTP insteated dTTP,the optimal dUTP concentration is approximately 10 times that of dTTP, a compensatory change in $MgCl_2$ was , therefore, necessary. Accordingly, we determined the optimal $MgCl_2$ again and found that the optimal concentration of $MgCl_2$ was 3.0 mM. For the negative control, DDW was used in place of sample for PCR and the false positive result due to amplicon carryover was not found.

False negative result, due to unknown inhibitors in the samples, may be detected by amplifying part of each sample spiked with 20×10^{-11} g pCHL2. By using the sample treatment protocol described in chapter III, all of the samples did not contain inhibitors.

The sensitivity of our PCR was 10^{-14} g of plasmid DNA when the product was analysed by gel electrophoresis and the sensitivity was increased to 10^{-15} g of plasmid DNA when the product was analysed by dot blot hybridization. Our sensitivity

corresponded well to the sensitivity of 10^{-15} g of total L2 DNA reported by Ostergarrd et al (31).

When we evaluated the efficiency of PCR for detection of *C. trachomatis* from endocervical specimens by comparing with the cell culture, we found that the PCR has a sensitivity of 100% and a specificity of 97.7 %. The two false-positive specimens were tested with a nucleic acid probe hybridization Kit (Gen-Probe™ Pace 2 system, Gen-Probe, Inc., San Diego,CA). Both of them were tested positive. Accordingly, the corrected specificity of the PCR was 100 %. This is in agreement with the results reported by Ostergaard,L. et al (31). Accordingly, we think that the PCR technique is a valuable tool for diagnosing genital *C. trachomatis* infection because of its high sensitivity and specificity. Since contamination due to amplicon carryover is the major problem of using PCR as diagnostic test , prevention of contamination must be considered. The technique that will resolve this problem should be used. One of this technique is performed by digestion of amplicon prior to PCR, using incorporation of dUTP instead of dTTP and uracil DNA glycosylase in the reaction.