CHAPTER 4

DISCUSTION

Alpha thalassemia is the common genetic defect throughout Southeast Asia (Wasi P., 1983). In Thailand, the incidence is in the range of 15 - 30 % (Winichagoon et al., 1992). The rightward α - Thalassemia 2 ($-\alpha^{3.7}$) is by far the most common single gene disorder world wide(Weatherall, 1986). Characterization of the molecular defects of $-\alpha^{3.7}$ kb deletion of α- thalassemia 2 has for a long time been achieved using restriction- enzyme analysis of genomic DNA followed by Southern blotting and hybridization with radio - labeled α - globin gene probes (Makonkawkayoon et al, 1993) . Both procedures are relative complex, time consuming and cumbersome. More over, they imply the use of radioactive reagents and for these reasons are not practical and suitable for most laboratories. The advent of the polymerase chain reaction (PCR) has revolutionized molecular biology and simplified detection of the most common variants of α - Thalassemia.

Polymerase chain reaction (PCR) analysis is a particularly usefull tool for the diagnosis of genetic disease. It can be applied to various molecular defects including point mutations, deletions and disease specific translocations for prenatal diagnosis, presymtomatic diagnosis or for carrier detection. In conjunction with PCR Allele Specific Oligonucleotide (ASO) hybridization (Saiki et al, 1986) or direct sequencing of the amplification product (Scharf et al, 1986) the molecular defects associated with many genetic disorder can be rapidly determined. Even when the genetic pathology has not been isolated, PCR of polymorphic markers in familial linkage studies can be diagnosis, or the refinement of the disease locus localization. Various strategies for the amplification of deletions and mutations have been developed, from those using a single pair of primers to detect small deletions such as the dalta 508 mutation in cytic fibrosis, to those requiring multiplex analysis (Kerem et al, 1989) for the detection of deletions spanning hundreds of kilobases as found in Duchene muscular dystrophy (Beggs et al,1990; Chamberlain et al,1988) .

Recently a method to screen for the most common α^0 - Thalassemia ($--^{SEA}$, $--^{MED}$, $-(\alpha)^{20.5}$ haplotype) by PCR amplification has been described by Bowden et al (1992). The method relies on the deletion event bringing 5' and 3' primers which are widely separated on the normal chromosome close enough together for amplification to proceed.

This study described a PCR base method for diagnosis of the most common from of α-thalassemia 2, rightward type ($-\alpha^{3.7}$). The sample can be characterized by comparing appearance and disappearance of PCR products on gel. Because of the PCR products from both pairs of primers are 1.76 kb for normal α2 gene and 3.7 kb deletion, the two separate reactions are run simultaneously for each DNA sample. The whole results were concluded in figure 17. It was extremely difficult to select the specific primers which provided the difference size of the amplification products for both two gene. Since the region between the α 1 and α 2 globin gene have a high degree of homology (98.5 %) so that the specific area for specific amplification is limited. In present study, 400 genomic DNA from Northern Thai populations, 67 (16.67%) were possessed alpha 3.7 kb deletion. When compared to the data provided by Hundrieser et al, 1988, the gene frequency of these deletion in samples from Chiang Mai was slightly greater than present study (17.9% VS. 16.67 %). In 1996, Lemmens et al. were reported the prevalence of $-\alpha^{3.7}$ in Chiang Mai populations. They were found that the gene frequency was 18.14 %. Since the samples of both reports were selected from healthy adult subjects at specific area only in Chiang Mai province, the value did not represent the whole populations. In this study, the samples were collected from 100 case of the Chiang Mai university's student , 200 from blood bank and 100 from pregnant women attending in Maharaj Nakorn Chaing Mai Hospital. All subjects lived in Northern part of Thailand from Nakorn Sawan to Chiang Rai . The distributions and the number of subjects in present study should give a more accurate results.

The reliability of the protocol was checked by identification of the α- globin genotypes of 20 normal DNA samples and 10 heterozygous for the deletion $-\alpha^{3.7}$ / αα (figure 18-20). The genotypes of all samples were identified

by radioactive hybridization with α1 specific probe (Makonkawkayoon et al,1993). The results have shown that these method can identify the α - thalassemia 2, rightward type ($-\alpha^{3.7}$) without false positive or false negative.

The optimization of PCR reaction such as the concentration of deoxy - nucleotide triphosphates (figure14), the primer annealing temperatures (figure 15), and the extension times (figure 16) were tritrated to provide the best results. A clear single band at 1.76 kb of amplification products had been observed without any complications neither nonspecific background bands nor the formation of primer - dimer.

The specificity and the fidelity of PCR increase at lower dNTP concentrations (Michael A ,1990). In this experiment , deoxynucleotide triphosphates concentrations were varied at 200, 300 ,400 μM . The best result was found at 200 μM without the formation of primer-dimer while at 300 and 600 μM, the primer-dimer had been observed (figure 14). The result suggested that the four dNTPs should be used at equivalent concentrations to minimize misincorporation errors.

Annealing is very critical step for the specificity of PCR products. The annealing temperature should be adjusted for each reaction. In this study, annealing temperatures were varied from 55°C to 70°C (58°C, 62°C and 68°C shown in figures 15), the best result was found at 68°C. The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of the amplification primers. An applicable annealing temperature is 5°C below the true Tm of the reaction are 88, 86 and 82 for A, B and C respectively , 68° C for primer annealing is adequate to provide the best result. The factors that decrease the annealing temperature in this study was dimethyl sulfoxide (DMSO). It have been reported that DMSO improved the amplification efficiency by reduced the secondary structures and melting temperature of DNA (Michael et al., 1995).

The extension time depends uponds the length and concentration of the target sequence and upon temperature (Micheal A, 1990). Primer extension temperature was performed at 72° C because this temperature was near optimal for extending primers on an M13-based model template (Michael A , 1990). The extension time was varied for 2.30 , 5 and 7 minutes. The expect band was present in all of these

time but the best had been observed in 2.30 minutes without non-specific bands. Although extension time of one minute at 72 ° C is considered sufficient for products up to 2 kb in length, but this protocol required more than the recommendation. It is because the higher melting temperature and longer length of primer require the longer time for primer extension.

This study not only characterized the alpha 3.7 kb deletion by PCR but also determined the hematological parameter. Although the hematological determination is widely used for routine screening test in populations where thalassemia is common but the reliability and the sensitivity of these parameters are not enough for the identification of all types of thalassemia. It has been reported that the hematological characteristic of the carriers state of alpha 3.7 kb deletion is not representative of the real phenotype (Galanello et al, 1998). This study tried to evaluated the hematological characteristic of this carriers. It was found that all hematological parameters range on the standard cut off of the normal value, and no statistical abnormalities can be detected ($P = 0.05$). The result suggested that the carriers state of alpha 3.7 kb deletion can not be identified by hematogical routine screening test. The PCR method is very useful for detection of these carriers, in order to evaluate and to control the spreading of alpha thalassemia in the future.