CHAPTER III

MATERIALS AND METHODS

Part I : Development of selective PCR for genotypic analysis of codon 215 AZT-resistant mutant.

1. The previously described selective PCR (Mitsuya H., et al.)

1.1 Plasmid control

Rhodes E-1 was used as a wild type 215 (Thr) plasmid and Pieler L-1 was used as a mutant 215 (Tyr) plasmid. They are consisted of 1689 bp of DNA fragment of *pol* gene in HIV-1 ligated to 2840 bp of pTZ 19R digested with EcoR I and Xba I. (4529 bp in total) [112]

1.2 Primer selection : *pol*-gene

DNA oligonucleotides were synthesized in a oligonucleotide synthesizer (Appendix I). The nucleotide sequences of the primers and their location in the HIV-1 genome are shown. The primer set AS62 and L1M were used in the first round PCR, the primer set WT 215/MT 215 and ANMER B were used in the nested or second round PCR which were specific for codon 215 mutation in HIV-1.

The designing of the primers were based on the single mutation principle for detection of AZT resistant mutant gene. The primer sets ANMER B and WT215/MT215 were specifically designed to amplify the nucleotides which code for the codon 215 wild type/mutant strain of the HIV-1, respectively. (Figure 1.)[113]

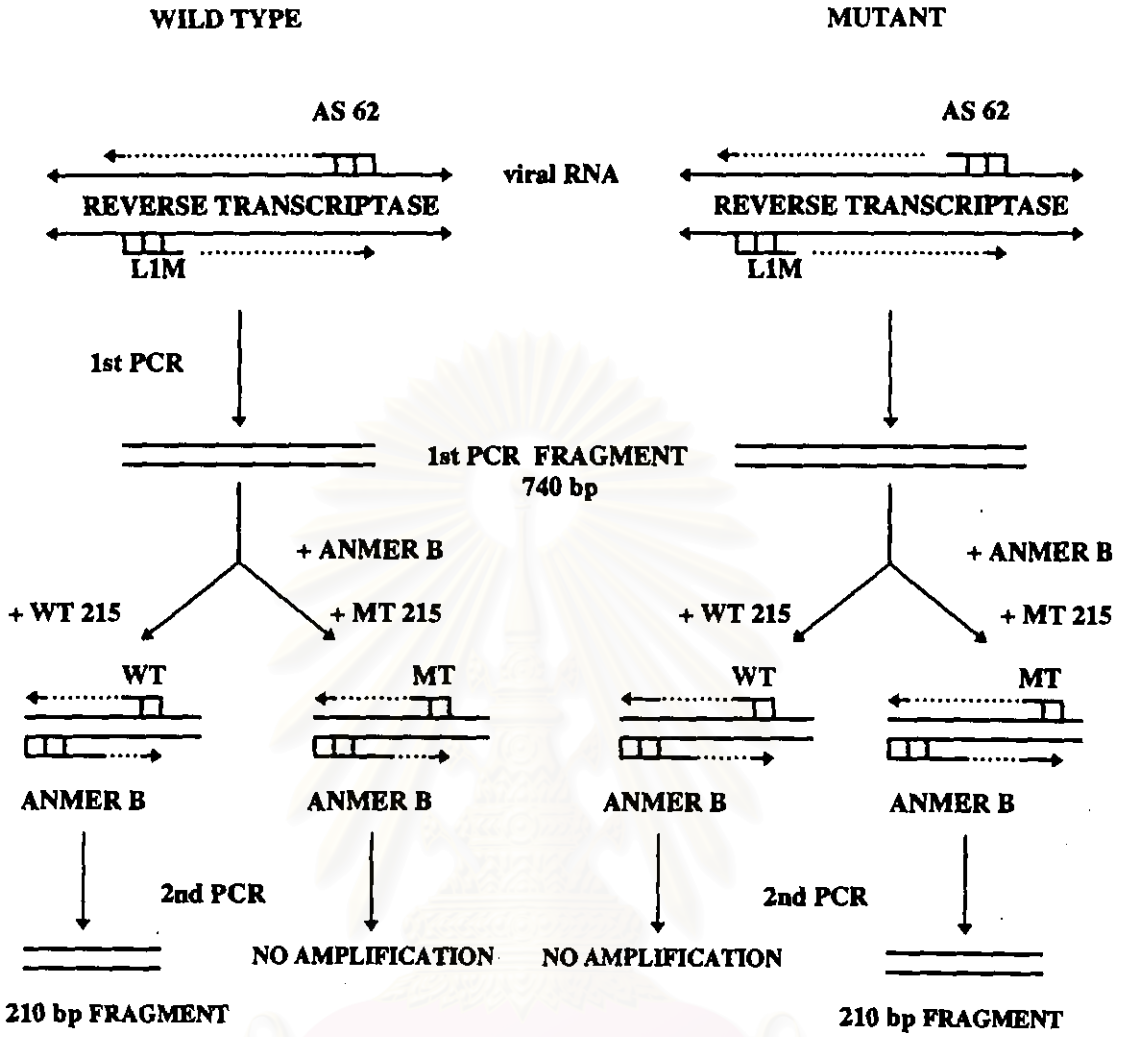

Figure 1. Schematic representation of selective PCR procedured used to detect mutation in the reverse transcriptase gene at codon 215 conferring AZT resistance

1.3 Analysis of codon 215 of *RT* gene by the previously described Selective PCR protocol

For analysis of codon 215 in the plasmid controls (wild type 215 and mutant 215), plasmid DNA were amplified using double PCR procedure. (Figure 1.) (113)

The PCR master mix with primer pair L1M and AS62 was prepared for the first round of amplification. A region encompassing codon 215 was amplified by using oligonucleotide primers (L1M and AS62) that annealed to the conserved sequences flanking this region. One microliter of plasmid DNA (wild type 215: Rhodes E-1/mutant 215: Pieler L-1) was amplified in a 50 µL reaction mix [1X PCR-buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each of dNTPs, 50 pmol of primer L1M (5'-TTG CAC TTT AAA TTT TCC CAT TAG-3') and 50 pmol of primer AS62 (5'-GGC TGT ACT GTC CAT TTA TCA GGA-3')]. *Tag* polymerase was added to the master mix (2.5 U per reaction) and dispense 48.5 µL of mixer per thin-walled MicroAmp tube. One microliter of plasmid DNA was added to each tube. Amplify the DNA in a programmable thermal cycle with a four-step profile: initial denaturation for 3 min at 95°C followed by a three-temperature cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C, for a total of 40 cycles in a GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk Conn.).

The second PCR step (selective amplification) was done with 2 µL of the first-amplified product in the reaction mixture described above. This step was duplicated in paralle for each sample; each reaction mixture contained primer ANMER B (5'-GGA TGG AAA GGA TCA CC-3') and either primer WT 215 (5'-ATG TTT TTT GTC TGG TGT GGT-3') to distinguish wild type residues, or primer MT 215 (5'-ATG TTT TTT GTC TGG TGT GAA-3') to distinguish mutant residues. The amplification procedure consisted of 35 cycles under the conditions in programmable thermol cycle with a four steps profile: initial denature for 3 min at 95°C followed by a three-temperature cycle of a 30 second at 94°C, a 30 second at an

annealing temperature at 56°C, and a 30 second at 72°C for a total of 35 cycles. The PCR product of 210 bp was obtained from amplification.

Analysis of Amplification products

Prepared a 1.5% agarose in TAE buffer containing 1 μg of ethidium bromide per mL. Electrophoretically separated the reaction products (10 μL per well) in TAE buffer, the bromphenol blue indicator should migrate about half the length of the gel. Visualized the amplified products (210 bp DNA fragment) on a short-wavelengh UV light transilluminator. Score each sample as wild type, mutant, or mixed type according to the presence of specific products and photographed.

The 100 bp DNA Ladder was used as a marker for the size estimates of the products.

2. Validation of RT-PCR assay in clinical samples

2.1 Study group

The stored plasma samples of HIV-1 infected patients whose the HIV-1 virus subtype had been characterized were subjected for anlysis. These were included 10 samples of subtype E and 10 samples of subtype B.

2.2 Sample preparation : RNA extraction

Viral RNA was extracted from plasma by treating 150 μL of plasma with 4 volumes of a lysis solution containing 5.75 M. GuSCN, 50 mM Tris (pH 7.5), 100 mM β-mercaptoethanol, and 1μL of poly (rA) per mL. The poly (rA) both facilitated the precipitation of viral RNA and reduced intersample variation. The lysates were incubated at 65°C for 10 min. The RNA was precipitated with 1 mL of isopropanol at room temperature, washed with 70% ethanol, and resuspened in 20 μL of DEPC-treated water. Sample were stored at -70°C until amplification.[114]

2.3 cDNA synthesis

Reverse transcriptase reaction was carried out as a single tube reaction with uninterupted thermal cycling by using the GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.) as follow. Reaction solution was prepared and dispensed in 20 μL aliquotes to thin-walled MicroAmp tube. The final reaction mixture contained the sample RNA, primer AS62 (5'-GGC TGT ACT GTC CAT TTA TCA GGA-3') at 50 pmol each, 200 μM dNTPs, 1.5 mM $MgCl_2$, and 1X RT-buffer. Added with 200 units of recombinant Moloney Murine Leukemia Virus Reverse Transcriptase (BRL) and 40 units of RNasin (Promega). The reaction was incubated at 37°C for 30 min.

2.4 PCR Amplification

For analysis of codon 215 of the reverse transcriptase (RT) gene, the RNA was reversely transcribed for cDNA. Then cDNA was amplified by using "double" PCR procedure described as in the 1.3 and 1.4 procedures. The plasmid wild type 215 and plasmid mutant were used as 215 a positive controls, and seronegative plasma RNA was used as a negative control.

2.5 Analysis of Amplification product

Ten microliters of the PCR product was analyzed as described in Part I (1.3).

<u>Part II</u> : Modified seminested RT-PCR to detect both HIV-1 subtype E and B

Since the primer L1M could not amplify the HIV-1 subtype E, It may suggest that in HIV-1 subtype E, the nucleotide sequence of the *RT* gene at this region (position 2554-2556) was varied from that subtype B. Therefore, primer ANMER B was used instead of primer L1M (in the first-PCR), and the process was carried out as described in part I (1.3).

Part III : Study the prevalence of AZT-resistant mutation at codon 215

A : Cross-sectional study

1. Study group

One hundred and fifty HIV-1 infected patients who came for regular follow-up at the HIV/AIDS Clinic of Chulalongkorn Hospital from December 1995 to August 1996 were recruited into this cross-sectional study. HIV-1 infection was identified by repeatedly reactive anti-HIV ELISA test which was subsequently confirmed by Western blot test or equivalent assays.

This study was approved by the Human Ethical Committee of the Faculty of Medicine, Chulalongkorn University. Patients were explained about the study objectives. One hundred and fourteen patients were males and thirty-six were femals. The ages ranged from 18 to 67 with a mean of 34.1 (S.D.=8.84). They could be classified into different stages according to CDC classification system,[115] namedly CDC stage II (asymptomatic HIV or persistent generalized lymphadenophathy (PGL) (N=25), CDC stage IV-A or HIV infection with constitutional symptoms or those conventionally called AIDS-related complex (ARC) (N=69) and CDC stage IV-C and IV-D or HIV infection with secondary infections disease and secondary cancers respectively or those conventionally called AIDS (N=56). Diagnostic criteria for PGL, ARC and AIDS were based on CDC classification system. (Appendix III) [115]

The subjects were enrolled due to the states of AZT treatment, and devided into 3 groups, 50 of each. Group I was AZT naive, Group II was AZT-experienced for less than 6 months, and group III was AZT-experienced for more than 6 months.

2. Specimen collection

Seven mililiters of blood was colleced from each subject by venepuncture into EDTA tubes, and the plasma was separated by centrifugation at 1,000 x g for 10 min within 3 hours after collection. The plasma samples were immediately frozen at -70°C until they were ready for processing and analyzed. Two milliliters of EDTA blood was also collected for CD4+ cell count by the use of flowcytometry.

3. Detection of AZT-resistant mutation at codon 215

To detect the presence or absence of AZT-resistant mutation at codon 215, the RNAs from all plasma specimens were extracted. Then RNA was reversly transcribed for cDNA. The cDNA was amplified by using modified seminested PCR as described in Part II.

B. Prospective study

1. Study group

Ten HIV-1-infected patients, selected from the previously described 50 patients who were AZT naive and had been followed-up after initiation AZT monotherapy for a duration of 5.2 to 13 months.

2. Specimen collection

The blood samples were collected at the time of initiating AZT treatment (designated as time zero), and at the consequent follow-up after 4 months of therapy. The samples were collected, stored, and analysed as described in part III A.