CHAPTER I

INTRODUCTION

1. BACKGROUND AND RATIONALE

The acquired immunodeficiency syndrome (AIDS) emerged during the 1980s as a major and burgeoning global health problem. It results from infection of certain components of the human immune system by the human immunodeficiency virus-1 (HIV-1). Currently, there is no known cure for AIDS. However, some progress has been made in understanding and treating this infection.

In recent years, significant progress has been made in the treatment of HIV-1 infected patient, mainly as a result of the development and clinical use of an increasing number of antiretroviral drugs. The US Food and Drug Administration (US-FDA) have approved three classes of drugs; 2 classes inhibit the HIV reverse transcriptase : nucleoside analogue RT inhibitors (NRTIs : AZT, ddI, ddC, d4T, 3TC and ABC) and non-nucleoside RT inhibitors (NNRTIs : NVP, DLV, EFV). The other class inhibits the HIV protease: protease inhibitors. A new nucleotide analogue RT inhibitors, tenofovir, has recently been approved.[1]

Although treatment of HIV-1 infected individuals with antiretroviral drugs is highly effective in increasing both duration and quality of life, the treatment failure can occur. Numerous factors contribute to the treatment failure, including poor compliance, lack of regimen potency and inadequate drug concentrations. However, resistance to antiretroviral drugs remains an important cause and consequence of treatment failure.

HIV drug resistance results from the rapid turnover of the virus population under the selection pressure of the antiretroviral therapy.[2] HIV has a half-life of 1 to 2 days coupled with the high mutation rate,[3,4,5] which is caused by the transcription error of reverse transcriptase. The main causes for the emergence of HIV drug resistance include suboptimal treatment or incomplete adherence to therapy.[6] Other causes are related to the pre-existence of drug resistance within HIV-1 quasispecies [4,7] and the transmission of HIV resistance at the time of the infection. [8,9]

At present, a number of resistance-associated mutations have been described. Resistance mutations to zidovudine (AZT or ZDV) appear sequentially under treatment pressure.[10] After the appearance of an unstable mutation K70R, the T215Y/F mutation can occur, often in association with M41L which result in partially resistant virus. If the treatment is continued, D67N, L210W, and K219Q/E mutations will have enhancing effect to the previous mutations and produce a high-level resistant virus. Thus, the first step toward significant resistance is the appearance of the T215Y/F mutation. For this reason, most investigators have looked preferentially to the T215Y/F mutation as a marker of AZT resistance. Recent study indicate that stavudine (d4T) can select for mutations typically associated with AZT resistance and also known as thymidine analog resistance mutations (TAMs).[11]

Of note, recent studies have found that these originally named as AZT-resistant mutations, in fact can be induced by other NRTIs : d4T, ddI, ddC, ABC. Thus they were currently called nucleoside analog mutations or NAMs. Furthermore, 4 NAMs or more mutation shown to cross-resistant within the NRTI class except lamivudine.[12]

More recently, a set of multi-nucleoside resistance (MNR) (A62V, V75I, F77L, F116Y, and Q151M) [13,14,15] was reported to have emerged under combination therapy using nucleoside analogues. These mutations, known as the Q151M complex, confer a much broader resistance pattern, referred to as multiple nucleoside analogue resistance. Because the Q151M substitution appears first and is thought to be crucial for subsequent development of multiple nucleoside analogue resistance, [13,14] the Q151M mutation has been considered as a marker of mutation for the multi-nucleoside resistance.

For the nonnucleoside reverse transcriptase inhibitors (NNRTIs), high-level resistance to individual compounds appears to develop rapidly within a few weeks of initiating monotherapy. The resistance frequently involved only single-point mutation and in many cases, leads to considerable cross-resistance to other NNRTIs. The most common mutations are K103N and Y181C.[16,17]

The identification of drug resistance becomes increasingly important. This can improve patient treatment by predicting drug failure and assisting with the choice of initial therapy when drug resistance is suspected or with the choice of alternative

treatment in the setting of treatment failure.[18] Recently, several phenotypic and genotypic assays for drug resistance have been developed. Phenotypic assays directly measure the degree of viral resistance but they are somewhat cumbersome, slow (weeks) and expensive. In contrasts, genotypic assays give faster results and avoid hazardous work with infectious material.

Genotypic resistance assay by HIV genomic sequencing is currently the reference procedure that provides information on all nucleotides on the regions being sequenced. However, this assay is expensive and laborious and thus unsuitable for resource limited countries. Simpler procedures are point mutation assays. For instance, selective polymerase chain reaction (PCR) and differential hybridization assays (e.g. line probe assay (LiPA)). After mutations at specific codons conferring resistance have been identified, selective PCRs with primers recognizing either the wild-type (WT) or mutant-type (MT) sequence at the codon of interest can be developed.

Selective PCR makes it possible to test large number of samples because of its simplicity and rapidly. However, this method becomes impractical when different positions of the viral genome need to be considered. Duplex selective polymerase chain reaction (PCR), based on the ARMS principle, becomes more suitable. It can detect two positions of interest in one reaction. The Amplification Refractory Mutation System (ARMS) has been successfully applied to the analysis of genetic mutation,[19] and has been used to apply for detection of HIV-1 drug resistance.[20] This procedure make primer more specific by introducing a series of additional mismatches at various sites into the primers.

In Thailand, most individuals are infected with Human Immunodeficiency Virus type 1 (HIV-1) subtype A/E (CRF01_AE), which is a different subtype from the reports of genotypic resistance of HIV-1 in North America and Europe where HIV-1 subtype B predominates. However little is known about the HIV-1 genotypic resistance in other part of the world, particularly in regards to other subtypes. Therefore, this study has evaluated the performance of the duplex selective PCR. The aim was to assess the usefulness of these assays for comparison of genotypic resistance (codon 103, 181, 151, and 215) between antiretroviral-naïve patients and antiretroviral-experienced of more than 6 months patients. This result may lead to a simple and cost-effective genotypic resistance testing for the epidemiological study of

HIV-1 drug resistance in Thailand. It may also be applicable for clinical uses in detecting NNRTIs and multi-nucleoside resistances.

2. RESEARCH QUESTIONS

Primary Question

How common of genotypic resistance among ART-Naive and ART-experienced > 6 months HIV-1 infected patients who have plasma HIV RNA > 1,000 copies/mL ?

Secondary Question

What is the prevalence of MNR (Q151M), NAMs (T215Y/F), and NNRTIs (K103N, Y181C) resistances in ART-naïve and ART-experienced HIV-1 infected Thais ?

3. LIMITATION

The important limitation of this study is that to obtain amplification from plasma require samples with HIV-1 RNA more than 1,000 copies/mL. The minority of mutations < 20% may not detectable.[93]

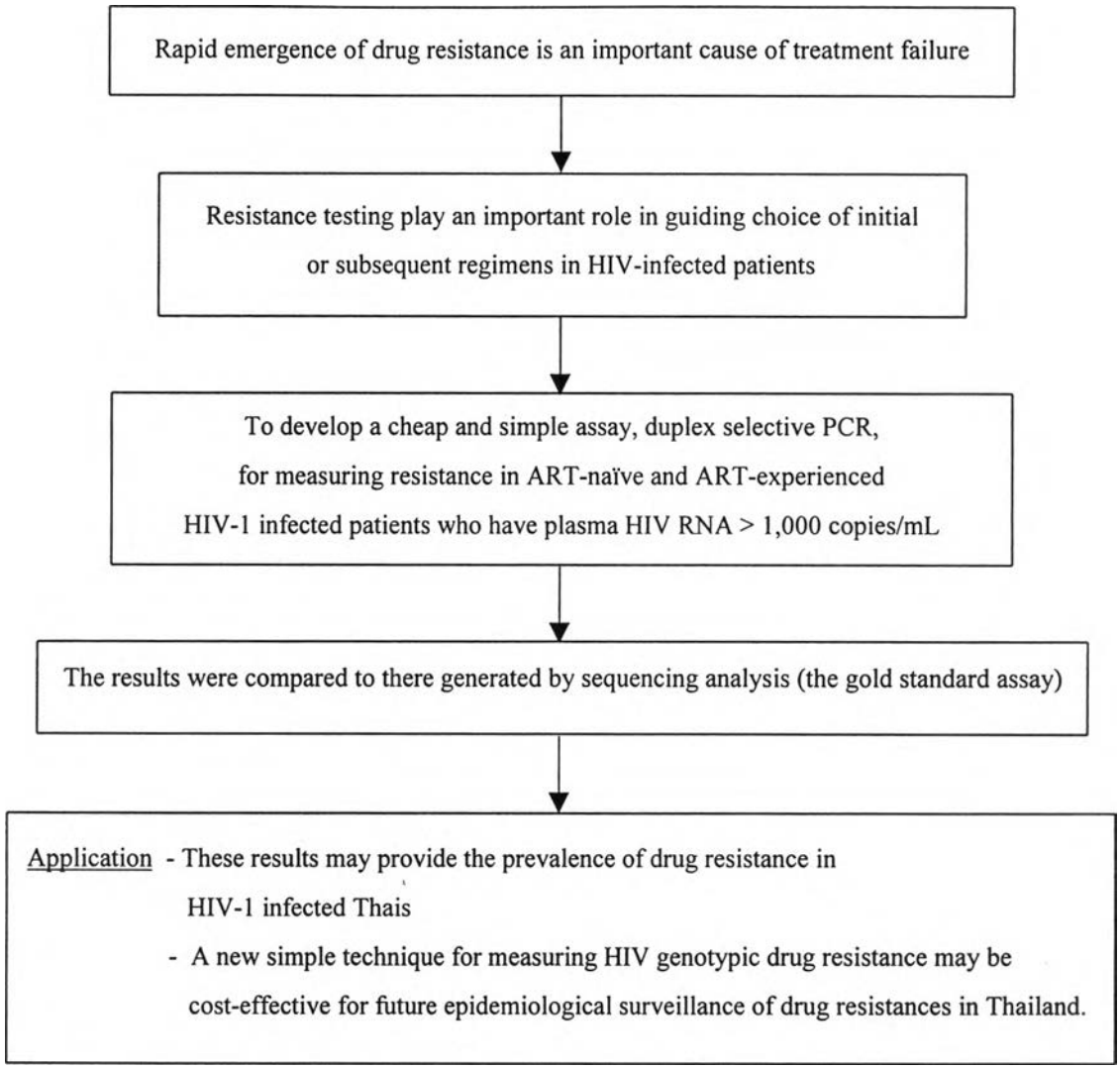
4. OBJECTIVE OF THIS RESEARCH

1. To compare the difference of genotypic resistance between ART-Naïve and ART-experienced > 6 months HIV-1 infected patients who have plasma HIV RNA > 1,000 copies/mL.

2. To assess the prevalence of MNR, NAMs and NNRTIs resistances in HIV-1 infected Thais.

3. The development of K103N / Y181C and Q151M / T215Y/F duplex selective RT-PCR in comparison to the sequencing analysis.

5. CONCEPTUAL FRAMEWORK



6. KEY WORDS

HIV-1

Resistance

Duplex selective PCR

NNRTIs, MNR, NAMs

Q151M, T215Y/F

K103N, Y181C

7. OPERATIONAL AND DEFINITION

Duplex Selective Polymerase Chain Reaction: a method whereby defines two segments of questionable nucleic acid by amplication in a single reaction with replicative denaturation and renaturation of the DNA helix in the presence of a DNA polymerase and synthetic oligonucleotides complementary to the segments in question.

8. EXPECTED BENEFIT AND APPLICATION

1. The difference in prevalences of genotypic drug resistance between ART-Naïve and ART-experienced > 6 months HIV-1 infected patients who have plasma HIV RNA > 1,000 copies/mL

2. The prevalence of HIV drug resistance in HIV-1 infected Thais.

3. To develop a new simple assay for measuring HIV genotypic drug resistance for laboratories that have the PCR facility.

4. The results of this study may help the clinicians to guide the choice of treatment in a more cost-effective way compared to the standard genotypic analysis.

9. RESEARCH METHODOLOGY

Subject Collection

Five mililiters of blood was collected from each of the two groups HIV-1 infected patients who have plasma HIV-RNA of more than 1,000 copies/mL

Group I : antiretroviral drug-naïve ; n=20

Group II : antiretroviral drug-experienced of more than 6 months ; n=25

Process of study

Step 1 : Primer Design

According to the published of drug resistance mutation in Los Alamos Drug Resistance Database, all PCR oligonucleotide primers were designed with the primer analysis software OLIGO version 4.1 (National Biosciences Inc, Plymouth, MN) and published sequence HIV-1 genotype E (CM240, U54771) information. The duplex selective primers were designed by the principle of ARMS.

Step 2 : PCR Standardization

To optimize the duplex selective PCR condition, the constructed primers were tested by using the following controls: HIV-1 (III_B) virions, wild type plasmid, and 151 mutant plasmid. If false positive mispriming effects occur, the primers will be redesigned.

Step 3 : Plasma HIV RNA Preparation for PCR Analysis

RNA was extracted from 1 mL EDTA plasma using lysis buffer (Guanidium isothiocyanate) and dissolved in 20 μL sample diluent.

Step 4 : cDNA synthesis

From the principle of RT-PCR, cDNA synthesis was performed in a total volume of 20 μL with 5 μL of RNA with the specific antisense primer OL.

Step 5 : PCR amplification

For analysis of changes in the RT gene, DNA was amplified by using a "double" PCR procedure. In the first step (pre-amplification) a region encompassing codon 103, 181, 151, and 215 was amplified by using primer (OU and OL) that anneal to conserve sequences flanking this region.

The second PCR step (duplex selective PCR) are divided into two groups by different condition

(1) K103N / Y181C duplex selective PCR

(2) Q151M / T215Y/F duplex selective PCR

The wild type plasmid and known mutant isolate (by sequence analysis) were used as positive controls, and nuclease-free water was used as a negative control.

Step 6 : Analysis of Amplification Product

Ten microliters of the PCR amplification product was electrophoresed in a 1.5 % agarose gel with 0.3 mg/mL ethidium bromide for 60 minutes at 60 V.

10. ADMINISTRATION AND TIME SCHEDULE

Phase	Process	Time Schedule											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Preparation phase	↔											
2	Primer design		↔	↔	↔								
3	Standardization of primers			↔	↔	↔	↔						
4	Subjects collection and preparation				↔	↔	↔	↔	↔	↔			
5	RNA extraction and cDNA synthesis					↔	↔	↔	↔	↔	↔		
6	Duplex selective PCR analysis						↔	↔	↔	↔	↔	↔	
7	Data and statistic analysis							↔	↔	↔	↔	↔	
8	Conclusion and writing the thesis										↔	↔	↔