GENOTYPIC SUBTYPING OF THE HIV-1 POLYMERASE GENE IN 30 NAÏVE PATIENTS FROM THAILAND

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Abstract. To investigate the subtype classification of the circulating virus strains among infected Thai patients with human immunodeficiency virus type 1 (HIV-1). A random population of patients who were HIV-1 antibody positive after two independent screening assays was selected. HIV RNA from plasma samples was reverse-transcribed and amplified with specific primers that annealed to conserve regions of the HIV-1 pol gene. Amplified products were sequenced directly by using an automated sequencer. The sequencing products represent about 1.2 kb of the pol gene from each patient and they were phylogenetically analyzed and compared to the corresponding pol sequences of the published HIV-1 sequences of known genotypes. Genotype E was found in 25 of 30 patients (83.3%), and 5 patients (16.7%) were HIV-1 genotype B. The result confirmed that HIV-1 subtype E is still predominant in Thailand. Genotype B is found frequently, but there have been no examples of genotype A. In concordance with the serotypic assay, which was previously reported using the V3-peptide enzyme immunoassay (V3-PEIA), the genotypic assay of subtype E was high, at 80% and 83.3% in serotyping and genotyping, respectively. These findings of two subtypes with low heterogeneity indicate that Thailand may be a desirable site for evaluating candidate HIV-1 antiretroviral drugs and vaccines. The mixture of subtype E and B' strains also offers the opportunity to study phenotypic differences between the two subtypes.

INTRODUCTION

Monitoring the transmission patterns of these virus variants (Lasky *et al,* 1997) is important, since Thailand has now become a major site for human immunodeficiency virus type 1 (HIV-1) infection, which occurs via various routes, such as sexual transmission or intravenous drug use (IDU) (Nelson *et al,* 1999; Winter *et al,* 1998). The diversity of HIV-1 strains may affect diagnostic tests and clinical practice; especially viral load measurements and sequencing based on genotyping for antiretroviral drug resistance testing (Haeselde *et al,* 1998). Moreover, the decreased susceptibility of non-B subtypes to antiretroviral drugs emphasizes the importance of the surveillance of HIV diversity (Haeselde *et al,* 1998). To date, two HIV-1 subtypes, B and E, have been isolated from Thai subjects based on the phylogenetic analyses of nucleotide sequences in the env gene (McCutchan *et al,* 1992; Ou *et al,* 1992). The immunologic relevance of genetic diversity, as it pertains to HIV-1 vaccine development, is not completely understood. *In vitro* and *in vivo* data suggest that HIV-1 subtypes B and E may be immunologically distinct (Mascola *et al,* 1994). It is unclear whether prophylactic vaccines need to be subtype specific. Nonetheless, knowledge of the incidence and prevalence of the HIV-1 subtype circulating in a target population will be an important component of any global strategy for the control of HIV-1. Tracking HIV by a subtyping assay can be conducted by either a genotypic or serological approach (Kusagawa *et al,* 1998). Using a serological assay, HIV-1 could be subtyped by the specific antibody in the serum or plasma obtained from infected patients. The antigen currently used for serological subtyping is the V3 region of envelope glycoprotein gp120. The V3 loop peptide enzymeimmunoassay (V3-PEIA) was recently introduced for prelimi-

nary HIV-1 subtyping (Chanbancherd *et al,* 1999). However, the antigenetically important, so-called V3 crown domain differs by only a few amino acids between subtypes. Therefore, cross reactivity may occur (Murphy *et al,* 1999). To overcome this problem, the genotyping assay of HIV-1 was utilized to identify HIV-1 subtypes in Thai population (Gaywee *et al,* 1996). In this study, a polymerase (pol) gene was chosen to sequence. This was due to the fact that the region not only enabled the determination of the distribution pattern of HIV, but also allowed pattern monitoring of resistant mutation to nucleoside reverse transcriptase inhibitors (NRTI), non nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) (Najera *et al,* 1995).

MATERIALS AND METHODS

Subjects

Thirty plasma samples from HIV-1 infected Thai naïve patients during 1999-2000 were collected. The CD4 and CD8 T-cell typing were performed using laboratory technique as described elsewhere (Dean *et al,* 1996). HIV-1 virion associated RNA in the plasma of patients was measured by the Amplicor HIV-1 monitor Assay (Roche Molecular Systems).

Serological determination of HIV-1 env subtypes by PEIA

The previously described report of HIV-1 subtyping using V3 loop peptide enzyme immunoassay (Subbarao *et al,* 1998) was used to compare with the result of sequencing of HIV-1 pol gene.

Heteroduplex mobility assay (HMA)

The previous result of HIV-1 env subtype were assigned by generation of heteroduplexs with analogous PCR product from set of reference HIV-1 strains of known env subtype, and examination of the mobility of these heteroduplexs after electrophoresis. The result of subtyping by heteroduplex mobility assay (Gaywee *et al,* 1996) was compared with the result from sequencing of HIV-1 pol gene.

DNA sequencing of C2-V3 region

The result from previous report of DNA sequencing analysis based on the C2-V3 region (Subbarao *et al,* 1998) was used compared with the result of sequencing of HIV-1 pol gene.

Molecular genotypic sequencing of HIV-1 pol gene

HIV-1 RNA extraction and cDNA synthesis: Viral RNA was extracted with Viral RNA Prep Kits (QIAamp, QIAGEN, Germany.) The extracted RNA was immediately reverse transcribed into cDNA by using Superscript One-Step RT-PCR reagent (Life Technologies, Gaithersburg, MD, USA) and pol gene specific primers (Winter *et al,* 1998). Direct DNA cycle sequencing of HIV-1 polymerase gene was performed by using dRhodamine dye-labeled dideoxyterminators assay (ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit, PE Biosystems) on ABI Prism 310 Automated DNA Sequencer (PE Biosystems). The electrophoregram fragments were assembled and manually edited by sequence navigator (PE Biosystems). Prior to phylogenetic analysis, acquired HIV-1 sequences were aligned by ClustalX with SIV-outgroup sequence and HIV-1 subtype E and B reference sequences. The alignment was then analyzed with the Phylogeny Inference Package version 3.57c (PHYLIP) (Felsenstein, 1993). Multiple sequence alignment was estimated phylogeny by 100 bootstrapping set of multiple sequence alignment. Phylogenetic distances of sequences within each isolate and among all isolates sequence were calculated with the two-parameter Kimura algorithm (DNADIST from PHYLIP). Dendograms were created by the neighbor-joining method. Tree diagram was plotted with Treeview program. Nucleotide sequence accession numbers. The sequences have been submitted to GenBank with accession no. AF191189 to AF191202, AF 191207 AF 193890 to AF 193895, AF 187307- AF 187309 and AF 240378 to AF240383.

After finishing genotypic study, the result of HIV-1 subtyping based on pol gene was used for definitive determination to which

Table1
Comparative result between the serological and molecular genotypic assays.

HIV subtype in Thailand	Serological assay	Molecular assay		
	V3-PEIA[a]	HMA[b]	Sequencing	
	n=214 (%)	env gene n=38 (%)	env gene[a] n=95 (%)	pol gene n=30 (%)
HIV-1 subtype E	175 (81.8)	32 (84)	77 (81.8)	25 (83.33)
HIV-1 subtype B	39 (18.2)	6 (16)	13 (18.2)	5 (16.67)

[a]Subbarao *et al,* 1998
[b]Jariyawat *et al,* 1996

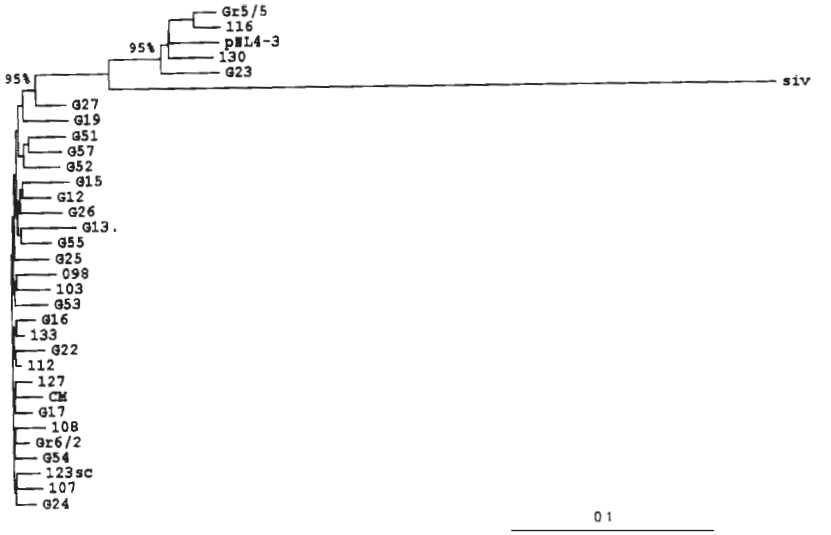

Fig 1–Phylogenetic trees, which were obtained for the pol gene from 30 naïve HIV-1 patients, show the correlation of HIV-1 sequences. The phylogenetic tree is constructed by neighbor-joining. Acquired HIV-1 sequences are divided into 2 clades by alignment with reference HIV-1 clade E (CM240) and B (pNL4-3). Outgroup is represented by SIV species.

the result obtained PEIA based on-, HMA based on- and sequencing analysis based on the env gene were compared.

RESULTS

Thirty sequences from antiretroviral-naïve patients were clearly subtyped by using molecular genotypic of the HIV-1 polymerase gene assay. Twenty-five (83.3%) sequences were closely clustered with the clade E reference subtype and 5 (16.7%) sequences were closely clustered with the clade B reference subtype. In a representative tree (Fig 1) containing 30 sequences from naïve infected Thai patients. The grouping for the 25 subtype E strains represented in this tree was supported by 95% of the bootstrap replicates; the subtype B strains grouped with a bootstrap supported of 95%.

DISCUSSION

The epidemiological study of HIV-1 transmission in Thailand is important, since extensive transmission and circulation of multiple subtypes have been reported (Lasky *et al,* 1997). Drug resistant mutations in each subtype express different gene patterns that respond to different drug therapy (Winter *et*

al, 1998). Thus, inappropriate drug treatment may not suppress or eradicate the virus in a patient's circulation. For the subtyping of HIV-1, a serological method has been available for sometime (Chanbancherd *et al,* 1999). This technique uses synthetic subtype-specific-peptide antigens that derived from an HIV-1 V3 loop of envelope gp120 glycoprotein to verify HIV-1 subtype (Murphy *et al,* 1999; Gaywee *et al,* 1996; Nkengasong *et al,* 1998). This assay has both advantages and disadvantages. The advantages of V3 peptide enzyme-immunoassay (V3-PEIA) are its relatively low cost, its technical simplicity, and the absence of requirements for special extensive equipment. Therefore, it has been widely used for preliminary subtyping.

The previous serological report using V3 PEIA showed that infected HIV-1 Thai patients were 80% and 20 % clade E and B, respectively (Chanbancherd *et al,* 1999; Nelson *et al,* 1999). From this study, HIV-1 subtyping by sequencing of the HIV-1 pol gene shows that 25 (83.3%) of the infected patients are HIV-1 subtype E and 5 (16.7%) are subtype B. Therefore, HIV subtyping using two methods; sequencing based subtyping of the HIV-1 pol gene region and previously reported, serological analysis using V3-PEIA (Gaywee *et al,* 1996), was concordant.V3-PEIA is the assay of choice for HIV-1 subtyping, however, it has a disadvantages in that only a few different amino acids between subtypes may cause cross reactivity. The principle neutralizing determinant (V3 loop) which may have implications for protein function as well as for serological assay based on this region and the inaccuracy of synthetic peptides in each laboratory to predict HIV-1 subtypes in individuals infected with different HIV-1 subtypes (A, B, C, D, F, G and H) varies considerably (Murphy *et al,* 1999; Gaywee *et al,* 1996). Due to the disadvantage of V3-PEIA, molecular subtyping of the HIV-1 gene is the assay that can use to verify an HIV-1 subtype in the Thai population.

Assays used to identify HIV-1 subtypes are not only V3-PEIA but also molecular genotypic assays are based on the HIV-1 env gene. Molecular HIV-1 subtyping has placed infected Thai patients into two clades, E and B, according to molecular studies involving env gene either sequencing (Kalish *et al,* 1995; Subbarao *et al,* 1998) or HMA (Gaywee *et al,* 1996). However, prior studies found that the inter-isolate divergence of the same subtypes for env sequences, which is in a determined geographical area, is higher than for pol sequences (Haeselde *et al,* 1995). The divergence between isolates for the same subtypes has been proposed as a marker of the time that specific viruses have been circulating and transmitting in a determined geographical area (Ou *et al,* 1993).

Recent studies reported that pol gene divergence did not correlate with the time of virus circulation as did env sequence divergence (Soto-Ramirez *et al,* 1996). The divergence of each subtype is independent of the time of existence of the isolates in that group. Finally, the heterosexual epidemic may have been spreading for longer than first thought, especially among female prostitutes (Soto-Ramirez *et al,* 1996). While it is currently easier to find differences or similarities among newly reported strains with env sequences, the expected evolution with increased divergence will require the use of more conserved regions (Soto-Ramirez *et al,* 1996). Therefore, in the continued the grouping of HIV-1 strains, the pol gene will offer a good option. HIV-1 subtyping by sequencing the HIV-1 pol gene may be one choice of an HIV-1 subtyping assay. However, in Thailand, the divergence of the env sequence has been low (Soto-Ramirez *et al,* 1996), the result from subtyping by sequencing the HIV-1 pol gene is consistent with the recent introduction of HIV-1 epidemiological data, which is based on the corresponding molecular genotyping by sequencing of the HIV-1 env gene (Soto-Ramirez *et al,* 1996). In addition, sequencing based genotyping of the HIV-1 pol gene can be used to manage antiretroviral therapy or monitor the emergence of antiretroviral drug mutations in HIV-1 strain (Najera *et al,* 1995).

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