SURVEILLANCE OF SUBTYPE AND GENETIC VARIATION OF THE CIRCULATING STRAINS OF HIV-1 IN THAILAND

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Abstract. Two HIV-1 strains, CRF01_AE and subtype B', were reported in Thailand during the early years of the epidemic. Recently, an intersubtype recombination of HIV-1 strain was found in Thailand. Eight-hundred and twenty-eight samples collected during years 1995-2004 from high-risk groups in Bangkok, northern, northeastern, and southern region of Thailand were studied. HIV-1 *env* nucleotide sequences were used for phylogenetic analysis of the circulating HIV-1 strain. By single HIV-1 region (*env)* genotyping, CRF01_AE was found in 97.3% and HIV-1 subtype B was found in 2.7%. A predominance of CRF01_AE was found in all geographic regions. Parallel analysis of the HIV-1 *gag* and *env* genes demonstrated that 2.1% and 4.0% of recombinant HIV-1 strains were found using p17 and p24 region sequences, respectively. The recombinant *gag* gene was also found in one southern isolate. Phylogenetic analysis of HIV-1 isolated from 20 provinces in 2002 suggested the northern and northeastern isolates were more related than the southern isolates which had the lowest genetic diversity of 0.13. The GPGQ V3 loop tip was also present in isolates from all regions. The molecular epidemiological data from this study may be useful for surveillance design as well as targeting prevention efforts. It also provides information regarding new antigenic regions of circulating strains responsible for the HIV-1 epidemic in Thailand.

INTRODUCTION

The diversity of human immunodeficiency virus type 1 (HIV-1) is expanding globally; primarily as a result of the low fidelity of the viral reverse-transcriptase (RT) and cellular RNA polymerase. These polymerases are responsible for high rates of substitution, insertion, and deletions during transcription events in the virus replication cycle (Mansky, 1998). There are 3 groups of HIV-1, groups M, N and O, of which, the main group (group M) is responsible for the pandemic. As of 2005, there have been 9 recognized subtypes (A-D, F-H, J-K), 2 sub-subtypes of HIV-1 (A1-A2, F1-F2), with 34 circulating recombinant forms (CRF) reported in the HIV Sequence Database (http://www.hiv-web.lanl.gov). While subtype B predominates in North America and Europe, HIV-1 CRF01_AE appears to be the cause of high proportion of infections in Thailand and Southeast Asia and seems to be largely confined to this area of the world.

The first case of AIDS in Thailand was reported in 1984. The patient was a Thai male who developed full-blown AIDS outside the country and returned home. More AIDS cases were reported during 1984-1988 but all of

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them were men who had sex with men (MSM) and had foreign partners (Phanuphak *et al*, 1985; Wangroongsarb *et al*, 1985; Limsuwan *et al*, 1986; Traisupa *et al*, 1987). In early 1988, HIV sero-surveillance studies in injecting drug users (IDUs) who attended methadone clinics in Bangkok were conducted, and the outbreak of HIV-1 in Thailand was first recognized among IDUs (Smith, 1990). The first HIV-1 molecular epidemiology study conducted in Thailand was reported in 1992 (Ou *et al*, 1992). Based on the analysis of C2-V3 nucleotide sequences, two HIV-1 genotypes in Thailand were identified. These 2 strains appeared independently in distinct high-risk groups, "genotype A" among heterosexuals and "genotype B" among IDUs; which were later named as subtypes E (and then CRF01_AE) and B, respectively. Further HIV-1 molecular epidemiology studies during year 1993-1994 showed that these 2 strains have been circulating in Thailand in distinct high-risk groups for more than a decade (Ou *et al*, 1993; Kalish *et al*, 1994).

Since 1995, HIV-1 CRF01_AE, which has been mainly found in sexually transmitted cases has caused an increasing proportion of new infections among IDUs in Thailand, which accounts for at least 80% of new infections in Bangkok and more than 90% in northern Thailand. Studies of HIV-1 subtypes among seroconverted individuals in Thailand from 1995 to 2000 reported that HIV-1 CRF01_AE accounted for more than 80% of cases in both IDU and sexual transmission groups (Subbarao *et al*, 1998). The long stable molecular picture for HIV-1 in Thailand appears to be entering a new phase of complexity when CRF15_01B was reported to be the first new circulating recombinant HIV-1 in Thailand in 2001 (Viputtijul *et al,* 2002). In 2003, a new recombinant virus between subtype C and CRF01_AE was reported in an HIV-1 infected Thai (Ramos *et al*, 2003; Tovanabutra *et al*, 2003).

As of December 2006, a total of 307,114 HIV infected cases have been reported in Thailand by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand (MOPH). During the past 10 years, heterosexual transmission has been the major route for developing of HIV-1 (83.9%) followed by IDU (4.4%). During the same period, the northern region of Thailand had the highest number of newly infected cases, followed by the central and northeastern regions.

The genetic variability of HIV-1 makes it possible to study the HIV epidemic within populations. Because HIV-1 strains evolve rapidly, only viruses that are epidemiologically related, such as founder viruses, will share sequence similarity that can be highlighted by analysis, such as phylogenetic analysis and signature sequence analysis. Such analyses have been used in various studies tracing global epidemiologic patterns (Nerurkar *et al*, 1996; Kuiken *et al*, 2000; Takebe *et al*, 2003; Sepherd *et al*, 2005; Tee *et al*, 2005). In this study, subtype determination and phylogenetic analysis were performed to study the molecular epidemiology of HIV-1 in Thailand.

MATERIALS AND METHODS

Study subjects

Eight-hundred twenty-eight samples were collected from asymtomatic and drug naïve HIV-1 infected individuals in Bangkok (BKK, n=88), northern (NO, n=295), northeastern (NE, n=200), eastern (E, n=2), and southern (SO, n=243) regions of Thailand from 1995 to 2004 through national surveillance programs. Of 828 samples, 786 were collected from the sexual transmission risk group, while 31, 9, and 2 samples were collected from IDU, perinatal risk groups, and blood donors, respectively.

All subjects in this study were asymptomatic with CD4+ count more than 200 cells/mm^3 and were antiretroviral drug naïve.

Specimen processing

Unclotted EDTA blood was collected after obtaining written informed consent from the HIV-1 infected individuals through the annual national surveillance program by the Bureau of AIDS, TB, and STIs, MOPH. Five milliliters of blood were collected in an EDTA vacutainer tube. The EDTA blood tube was centrifuged at 1,800 rpm for 5 minutes to separate blood cells from plasma. Plasma was removed and stored at -70ºC for HIV-1 viral RNA extraction.

Amplification of HIV-1 gene regions

HIV-1 viral RNA extracted using QIAamp spin columns (QIAGEN Gmbh, Hilden, Germany) was performed according to manufacturer's instruction.

The C2-V4 region of the *env* gene, and the p17 and p24 regions of the *gag* gene were amplified by RT-PCR using a One step Superscript RT-PCR with platinum Taq DNA polymerase (Invitrogen, CA, USA) and nested-PCR. The primers for HIV-1 gene amplification are shown in Table 1. The RT-PCR reaction mixture consisted of 25 µl of 2X reaction mix, 1 µl each of 10 pmol/µl outer primer, 2 µl of superscript RT-PCR with platinum Taq DNA polymerase, 10 µl of HIV-1 viral RNA and 11 µl of deionized water. The reaction mixtures were transferred to a 2400 DNA Thermal cycler (Applied Biosystem, CA, USA). The cycles consisted of a reverse transcription step at 55ºC for 30 minutes then inactivated RT enzyme at 94ºC for 15 minutes and 35 cycles of 94ºC denaturation step for 1 minute, a 55ºC annealing step for 1 minute, and a 68ºC extension step for 3 minutes and the final extension at 68ºC for 5 minutes.

For Nested PCR, 5 µl of first round RT-PCR product was mixed into the reaction tube which consisted of 5 µl of 10X PCR buffer, 3 µl of 25 mM $MgCl_2$, 6 µl of 10 mM dNTP (Promega, WI, USA), 3 µl each of 10 pmol/µl inner primers, and 2.5 U of *Taq* DNA polymerase (Promega, WI, USA). The amplification cycles were carried out at 94ºC for 5 minutes followed by 35 cycles of a 94ºC denaturation step for 1 minute, 55ºC annealing step for 1 minute, and 72ºC extension step for 3 minutes and final extension at 72ºC for 7 minutes.

HIV-1 nucleotide sequencing and analysis

A 530-base pair, 500-base pair, and 800-base pair RT-PCR products of C2-V4 of *env* gene, p17 and p24 of *gag* gene, respectively, were purified using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). DNA cycle sequencing was done bidirectionally using BigDye Terminator Ready Reaction Mix (Applied Biosystem, CA, USA) with nested primers for each region of the HIV-1 gene. The reaction mixture contained the template DNA of approximately 100-250 ng, 4-8 pmol/µl of sequencing primers and 4 µl of terminator dye (containing deoxynucleotides, dye labeled dideoxynucleotides, a polymerase enzyme and buffer system). When the reaction was terminated, the product was labeled with the specific dye corresponding to that specific nucleotide. The excess dye-labeled dideoxynucleotide was eliminated by precipitating with 95% ethanol in a 1.5 microcentrifuge tube. After the fluorescent labeled ssDNA was completly dried, the sequencing was carried out by an ABI 310 automatic sequencer.

A chromatogram of HIV-1 nucleotide sequences was performed using Chromas version 1.45. Lasergene Software version 5.0 (DNASTAR, WI, USA) was used to edit and translate the HIV-1 nucleotide sequences. The edited nucleotide sequences were aligned using ClustalX (Thompson *et al*, 1997). Phylogenetic analysis, statistical robustness of the neighbor-joining tree with F84 evolutionary model and bootstrap test of 500 replicates, were performed with PHYLIP 3.63 software package for Microsoft Windows (distributed by the author, Department of Genome Sci-

ences, University of Washington, Seattle) to determine the subtype of HIV-1 (Felsenstein, 1993). Known subtype HIV-1 reference strains were included in the analysis. The phylogenetic tree was constructed by TREEVIEW, version 1.6.6 (distributed by Roderic DM, Page 2001 in: http://taxonomy.zoology.gla.ac.uk/rod/rod/html).

Bootscanning analysis contained in SimPlot version 3.2 (Lole *et al,* 1999) was used to identify and to determine the significance of recombination breakpoints. A neighbor-joining algorithm with 500 replicates, using sliding windows of 200 nucleotides with an overlap of 50 nucleotides, was applied to the analysis. The background sequences that were used included CRF01_AE (CM240), subtype B (MN), and subtype C (96BW05), all of which are reference strains available at the HIV Sequence Database website (http://hiv-web.lanl.gov). The breakpoint was determined by a significant ($p<0.001$) difference in the ratio of informative sites for each HIV-1 subtype on either side of the breakpoint, as assessed by the Maximum Likelihood Chi-squared (Martin *et al*, 2005).

HIV-1 nucleotide sequence accession numbers

HIV-1 nucleotide sequences in this study have been submitted to GenBank, the US National Institutes of Health (NIH) genetic sequence database, under accession number AY005164, 66, AF373038-40, AY005172, AY005168, AF373041, AY005174,76, AY005170, AF373042-3, AF449824-50, AF449894-908, AF449883, AF449936-63, AF449864-79, AF449884-7, AF449880, AF449888-9, AF449912-9,23,27-34, AF449993, AF449805-6, AF449922,24-6, AF449854-8, AF449799-800, AF449808-20, AY243225-36, AY243241-2, AY243180-94, AY243195-216, AY243237-40, AY248123-33, AY248134-8, AY621260, AY621237-54, AY621264-70, EF063390, AY248113-22, AY248107-12, AY248139-55, AY243217-24, AY243135-62, AY243164-8, AY243311, AY243171, AY242288, AY248288, AY248040-4, 51-7, 88-92, AY243173, AY248058-60, AY248066-77, AY248093-106, AY243174-9, AY248045-50, AY248061-5, AY248078-87, AY621174-83, AY621198, AY621286-92, AY243308-10, AY243170, AY248156-69, AY248213-9, AY243283-92, AY248199-208, AY248233-7, AY248252-60, AY248275-87, AY248039, AY248181-8, AY248209-12, AY248238-40, AY248261-5, AY243244-8, AY243266-82, AY243293-301, AY243249-65, AY248241-6, AY243302-7, AY248189-98, AY248221-32, AY248248-51, AY248266-74, AY621261-3, EF063393-438, EF063391-2.

RESULTS

HIV-1 subtype determination using C2-V4 region of *env* gene

Among 828 HIV-1 nucleotide sequences, 806 (97.3%) sequences were HIV-1 CRF01_AE and 22 sequences (2.7%) were HIV-1 subtype B.

The analysis of HIV-1 circulated in each geographic region in Thailand demonstrated that CRF01_AE was predominant in all regions. HIV-1 CRF01_AE was found at 95.5, 99.3, 98.0, 95.1, and 100% in Bangkok, northern, northeastern, southern, and eastern regions, respectively. HIV-1 subtype B was found at 4.5%, 0.7%, 2.0%, and 4.9% in Bangkok, northern, northeastern, and southern regions, respectively. There was no HIV-1 subtype B found in the eastern region for this study.

HIV-1 CRF01_AE was found at 95.9% among males and 96.6% among females in this study; HIV-1 subtype B was found at 4.1% and 3.4% among males and females, respectively (Table 2).

Parallel phylogenetic analysis of HIV-1 *gag* and *env* gene

HIV-1 *gag* gene (p17 and p24 regions) of the corresponding samples were used in parallel phylogenetic analysis to identify discordant subtypes. The phylogenetic analysis model for *gag* gene analysis was the same as

those used for the *env* gene.

Parallel phylogenetic analysis of 193 nucleotide sequences of p17 region which corresponded to C2-V4 region sequences reveals that 189 samples (97.9%) had the same subtype in both regions, while the remaining 4 (2.1%) had a discordant subtype. Among concordant subtype samples, 10 samples (5.3%) were subtype B and 179 samples (94.7%) were CRF01_AE.

Seventy-six nucleotide sequences of the C2-V4 region, which corresponded to the p24

Table 1
Primers for HIV-1 gene amplification and direct nucleotide sequencing.

Name	Primer sequence (5´-3´)	Location in HXB2
JH35	CACTTCTCCAATTGTCCITCA	6371-6392
JH44	ACAGTRCARTGYACACATGG	7891-7871
JH33	CTGTTIAATGGCAGICTAGC	7002-7021
JH48	RATGGGAGGRGYATACAT	7540-7524
Whole gagO1	GCGGAGGCTAGAAGGAGAGA	769-788
Whole gagO2	GCTCTCCTCCGATTCTTACTG	5177-5197
P17I1	GAGAGATGGGTGCGAGAGCG	785-804
P17I2	GTACCATTTGCCCTTGTGC	1219-1201
P24I1	GTCAGCCAAAATTACCCT	1171-1188
P24I2	GTTGTGCTTGGCTCATTGCCTC	1904-1182

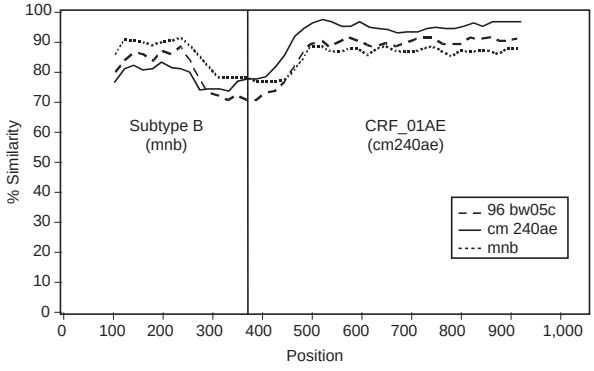
The JH primer set was used to amplify C2-V4 region.
WholegagO1/O2 and p17I1/I2 were used to amplify the p17 region.
WholegagO1/O2 and p24I1/I2 were used to amplify the p24 region.

Table 2
Percentage of HIV-1 subtypes found in Thailand by phylogenetic analysis of C2-V4 nucleotide sequence.

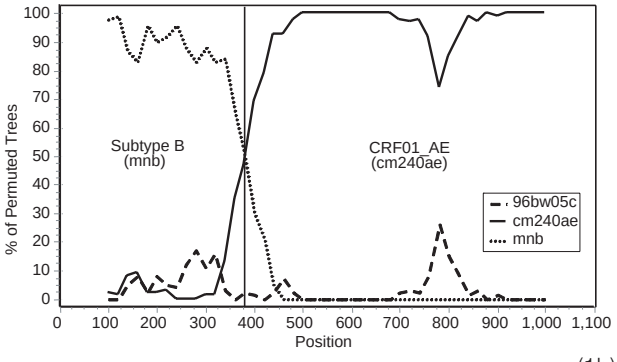
	%CRF01_AE (no./total)	%Subtype B (no./total)
By year		
1995-1996	100 (10/10)	0 (0/10)
1997-1998	100 (48/48)	0 (0/48)
1999-2000	97.8 (132/135)	2.2 (3/135)
2001-2002	96.9 (567/585)	3.1 (18/585)
2003-2004	98.0 (49/50)	2.0 (1/50)
By geographic region		
Bangkok	95.5 (84/88)	4.5 (4/88)
North	99.3 (293/295)	0.7 (2/295)
Northeast	98.0 (196/200)	2.0 (4/200)
South	95.1 (231/243)	4.9 (12/243)
East	100 (2/2)	0 (0/2)
By gender		
Male	95.9 (210/219)	4.1 (9/219)
Female	96.6 (375/388)	3.4 (13/388)

Table 3
Phylogenetic classification of HIV-1 isolates with discordant subtype.

	Subtype designation				Demographic data		
No.	p17	p24	Pol	C2-V4	Year	Area	Gender
NO350	01_AE	01_AE	-	B	2002	Phitsanulok	Female
SO469	01_AE	01_AE	B	B	2002	Nakhon Si Thammarat	Female
SO868	B	01_AE	B	B	2002	Nakhon Si Thammarat	Female
NE1036	01_AE	01_AE	B	B	2002	Nong Khai	Female



(1a)



(1b)

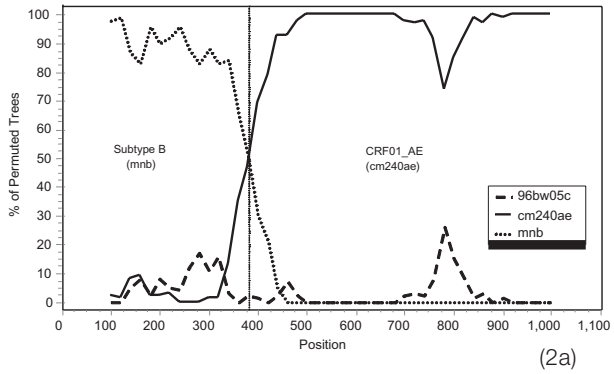
Fig 1–Similarity plot (1a) and BootScanning plot (1b) of sample No. SO868 which contained B/01_AE recombinant genome in the *gag* gene. The regions of subtype B and CRF01_AE were depicted in different line patterns (dots and solid). The bootstrap values were plotted for a window of 100 bp moving along the alignment. The verticle lines show the B/CRF01_AE recombinant breakpoint.

region, were analyzed. Of the 76 sequences, 73 (96.0%) were concordant with the C2-V4 region and 3 (4.0%) were discordant. There was also 1 sample which was discordant between the p17 (subtype B) and p24 (CRF01_AE) regions of the *gag* gene (Table 3).

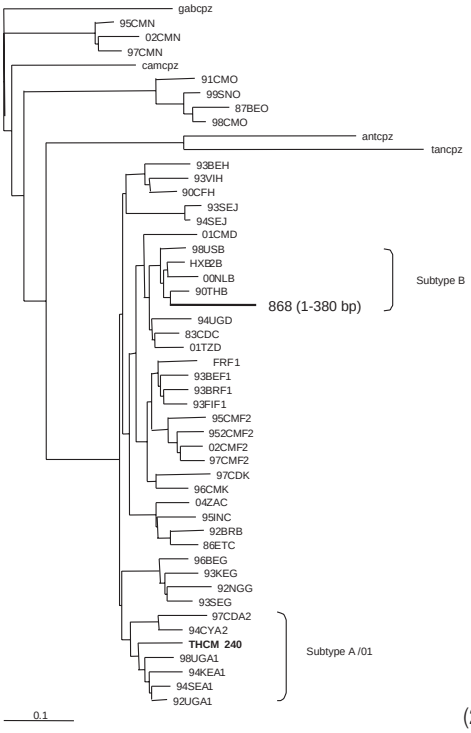
Samples discordant between the *gag* and *env* genes were further analyzed by phylogenetic analysis of the partial reverse-transcriptase (RT) region nucleotide sequences of the *pol* gene. A neighbor-joining tree of the partial RT region nucleotide sequences of 600 base-pairs of 3 subtype discordant samples and HIV-1 reference strain nucleotide sequences demonstrated that all 3 samples contained the HIV-1 subtype B genome in the RT region of the *pol* gene.

Recombinant HIV-1 *gag* gene analysis

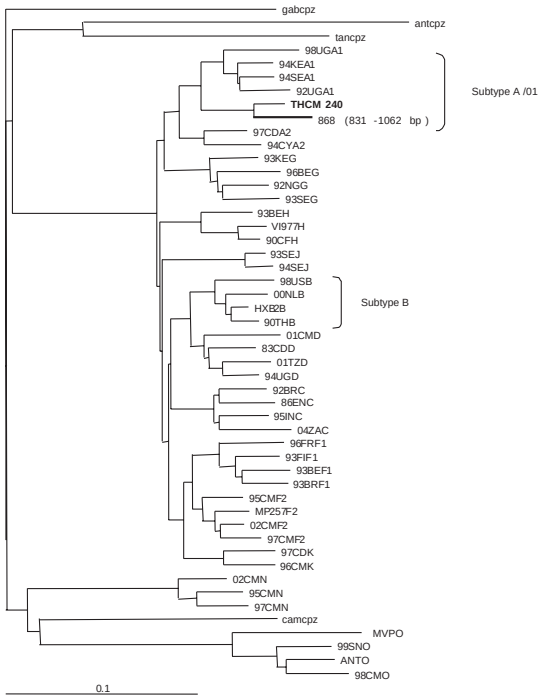
One sample (SO868), which contained HIV-1 recombinant genome in the *gag* gene, was further identified for recombination breakpoint by bootscan analysis using SimPlot version 2.5. Nucleotide sequence position 1-380 of HIV-1 *gag* gene had 90% similarity with HIV-1 subtype B reference strain MN while position 381-1062 had 98% similarity with CRF01_AE reference strain CM240 by SimPlot. The breakpoint where recombination of 2 subtypes occurred was identified at the nucleotide position 380 bp of *gag* gene with bootstrap support of 47% permuted tree (Fig 1). The phylogenetic tree of the *gag* gene nucleotide sequence from positions 1-380 and



(2a)



(2b)



(2c)

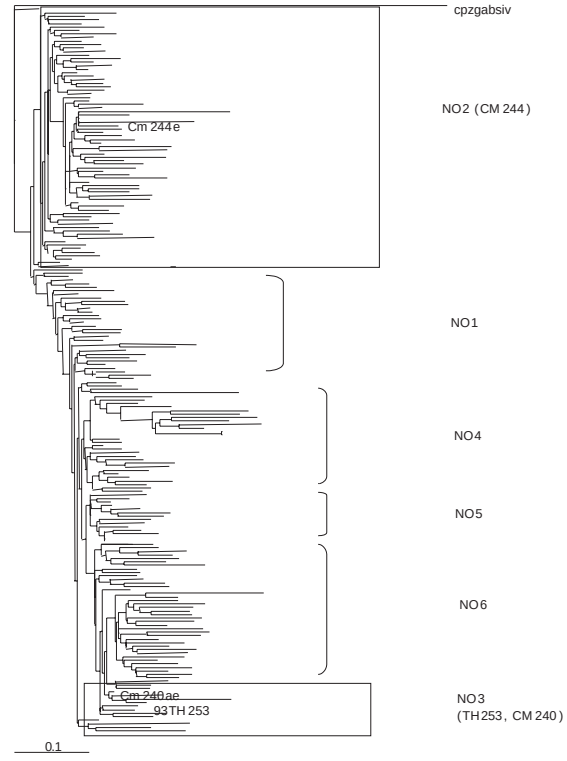
Fig 2–Exploratory tree analysis of *gag* gene nucleotide sequence position 1-830 (2b) and position 831-1062 (2c). The pausible recombination breakpoint was identified and the HIV-1*gag* gene was then divided into 2 segments (2a). Each segment was used in separate phylogenetic analyses based on the neighbor-joining method to confirm the subtype origin of the segment.

381-1062 was separately constructed to confirm recombination breakpoint found by bootscan (Fig 2).

Phylogenetic clustering of CRF01_AE circulating in Thailand

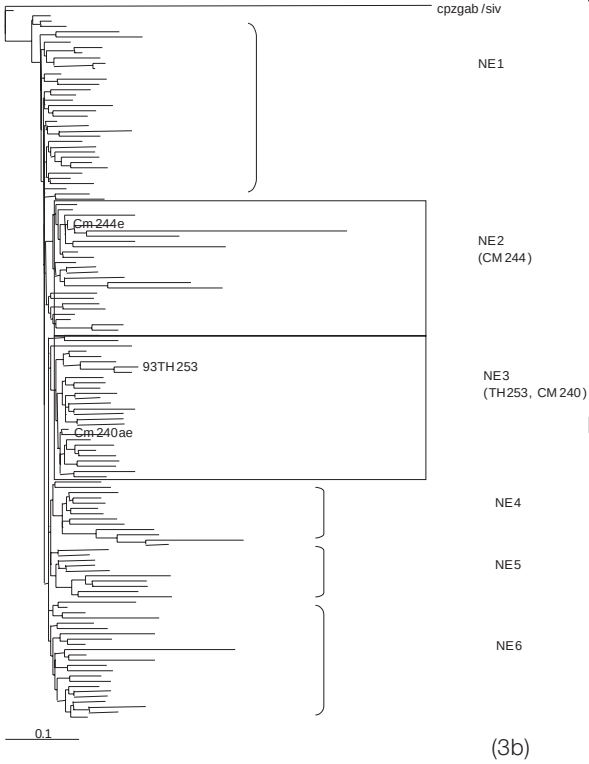
HIV-1 CRF01_AE isolates collected from recently seroconverted individuals in northern, northeastern, and southern regions of Thailand were used to analyze the cluster of CRF01_AE strains. These individuals were asymtomatic, contracted HIV heterosexually, and did not receive any anti-retroviral treatments. Five hundred sixty-seven CRF01_AE *env* (C2-V4) nucleotide sequences isolated in 2002 were used for a cluster study of CRF01_AE strains circulating in Thailand.

All phylogenetic trees of CRF01_AE nucleotide sequences from each geographic region (NO, NE, SO tree) demonstrated the same patterns of phylogenetic clustering (Fig 3). Six phylogenetic clusters were identified among these sequences. These clusters were



(3a)

(3c)



(3b)

Fig 3–Phylogenetic trees of CRF01_AE isolated from northern (3a), northeastern (3b), and southern (3c) Thailand. All CRF01_AE isolates in each region clustered together without geographic distinction. The phylogenetic trees were rooted with SIVcpz. All phylogenetic trees shared the same pattern of clustering. Two clusters, which the circulating strains are clustered with the reference strain, are indicated. The HIV-1 CRF01_AE reference strains, CM244, CM240, and 93TH253, are indicated.

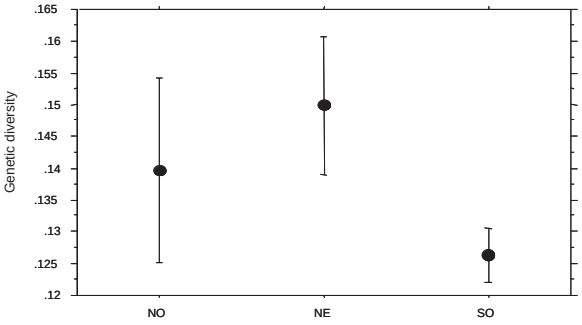


Fig 4–Mean genetic diversity of CRF01_AE circulated in northern, northeastern, and southern Thailand. The southern isolates had the lowest genetic diversity ($p<0.05$) with mean genetic diversity of 0.14 while there was no difference in the mean genetic diversity of CRF01_AE circulating in northern and northeastern Thailand.

designated as NO1-6, NE1-6, and SO1-6 cluster for clusters from NO, NE, and SO trees, respectively. There was one cluster which CRF01_AE sequences clustered with 2 Thai reference strains, TH293 and CM240, isolated in the years 1993 and 1990, respectively. The other major cluster of these 3 phylogenetic trees was a cluster of CRF01_AE sequences with another Thai reference strain, CM244, isolated in the year 1990. The other 4 clusters consisted of CRF01_AE sequences from provinces in each region clustered together.

The phylogenetic tree for CRF01_AE sequences isolated in the year 2002 was constructed with a Maximum-likelihood method which demonstrated that there was no geographic distinction among these sequences. HIV-1 nucleotide sequences isolated from Bangkok in 2000 were also analyzed with HIV-1 nucleotide sequences from each region. The phylogenetic analysis of HIV-1 Bangkok isolates and HIV-1 NO, NE, and SO isolates demonstrated that there was also no geographic distinction among HIV-1 isolates in Thailand. HIV-1 sequences of Bangkok isolates clustered with HIV-1 sequences isolated from other regions.

Genetic diversity of CRF01_AE circulating in Thailand

The maximum likelihood distance of each group was computed to study the average genetic diversity of NO1-6, NE1-6, and SO1-6 clusters. Mean genetic diversity (±SD) of CRF01_AE circulating in northern, northeastern, and southern Thailand were 0.140±0.36 (0.109-0.201), 0.150±0.22 (0.117-0.194), and 0.126±0.10 (0.117-0.146), respectively (Fig 4). Comparing the genetic diversity of the geographical trees confirmed clustering in each tree that was different in genetic diversity among the 6 clusters in the NO ($p<0.0002$), NE ($p<0.0001$), and SO ($p<0.0001$) trees. CRF01_AE circulating in southern Thailand had the lowest genetic diversity ($p<0.05$) compared to that circulating in other regions of the country. There was no difference in mean genetic diversity among the CRF01_AE circulating in northern and northeastern Thailand ($p<0.05$).

Signature sequence pattern

Signature sequence analysis of each cluster in the NO, NE, and SO trees was done using HIV-1 CM244 strian, which is a CRF01_AE reference strain, as a background for the analysis. CRF01_AE circulating in the northeast (NE6 cluster) had the highest number of variable positions (15) compared to CM244, while those circulating in the south (SO4 cluster) had the lowest number of variable positions (4). The variable position identified by signature sequence pattern analysis was consistent with the mean genetic diversity that CRF01_AE circulating in the south had low genetic diversity. Among 6 clusters in the NO tree, NO4 cluster had the highest number of variable amino acid positions. For the southern isolates of CRF01_AE, there were 2 clusters that had the highest number of variable amino acid positions (9), the SO1 and

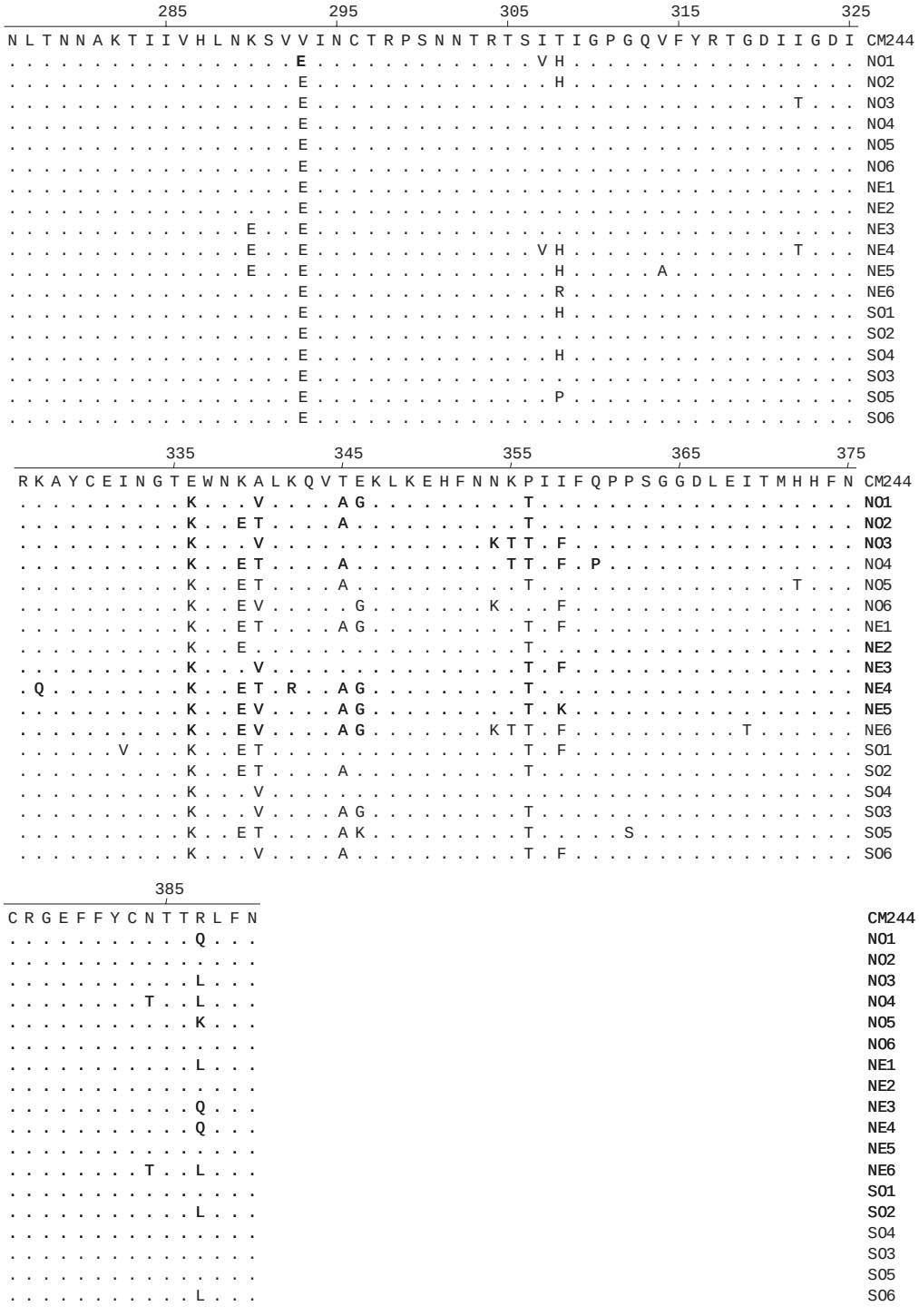


Fig 5–Signature sequence patterns of CRF01_AE amino acid sequences of NO1-6, NE1-6, and SO1-6 clusters compared with reference strain CM244. Dots indicate similar amino acid sequences between each cluster and the reference strain. Amino acid position in the alignment was numbered according to the HXB2 numbering engine for the gp160 region of the *env* gene.

SO5 clusters. These 2 clusters shared the same major population of CRF01_AE isolated from southern coastal provinces in the Gulf of Thailand. GPGQ was the predominant V3 loop tip pattern in all clusters.

N-linked glycosylation prediction

There were 6 acceptor sites for N-linked glycosylation reported for the C2-V4 region sequence of the CM244 reference strain at amino acid positions 275, 289, 295, 301, 333, and 384. N-GLYCOSITE was used to predict N-linked glycosylation sites on amino acid sequences of CRF01_AE in 6 phylogenetic clusters of NO, NE, and SO trees. All 6 N-linked glycosylation sites found in CM244 were also found in 14 clusters of CRF01_AE isolated in 2002. Clusters NO4 and NE6 did not have N-linked glycosylation at position 384 by amino acid subsitution from N to T. Two additional N-linked glycosylation sites at amino acid positions 338 and 353/354 were predicted from CRF01_AE isolated from all regions. The additional sites at amino acid position 338 were found in 8 clusters (NO2,4,5; NE1,4; SO1,2,5) by amino acid substitution from A to T at position 340. Another additional site at amino acid position 353/354 was found in 16 clusters. It was caused by amino acid substitution from P to T.

DISCUSSION

In this study, phylogenetic and signature sequence analyses were used to trace the evolution and epidemiology of the circulating HIV-1 strains in Thailand.

The C2-V4 region of HIV-1 *env* gene nucleotide sequences from infected individuals in major epidemic areas in Thailand were used to study HIV-1 subtypes. The predominant subtype of HIV-1 circulating in all geographic regions of Thailand during the years 1995-2004 was CRF01_AE (97.3%). This finding is consistent with a previous report in 1995 when there were no recombinant strains (Kalish *et al*, 1994).

Full length analysis of HIV-1 isolate CM237 previously reported to be subtype B by one region genotype, revealed this isolate had a recombinant genome. The discordant subtype between *gag* and *pol* genes indicates that HIV isolate CM237 was a CRF01_AE/B recombinant strain (Magiorkinis *et al*, 2002). Thus, parallel phylogenetic analysis, an analysis of corresponding HIV-1 *gag* and *env* genes, was used to screen recombinant genomes of HIV-1 strain in this study. By parallel phylogenetic analysis, 2.1% and 4.0% of HIV-1 strains with discordant subtypes were found by p17 and p24 regions of HIV-1 *gag* gene analysis, respectively. The percentage of subtype discordance found in this study was lower than that reported during the same period from a cohort study of IDU in Bangkok during 1995-1996 and a prime-boost phase III vaccine trial during 1998-2001 (Ramos *et al*, 2003; Watanaveeradej *et al*, 2006). The lower percentage of subtype discordance found in this study may come from analysis of fewer regions of the HIV-1 genome because full length genome analysis was performed in previous studies. The reverse transcriptase region (RT) of the HIV-1 *pol* gene was used to analyze discordant subtype of isolates that have a recombinant genome. However, no subtype discordance was found between the *env* and *pol* genes in this study, which may be due to the lower genetic diversity of the *pol* gene (Sirivichayakul *et al*, 2001). The discovery of recombinant strains of HIV-1 in this study by parallel phylogenetic analysis indicates that current subtype screening by simple genotyping may not be sufficiently effective to capture the increasing complexity of recombinant genomes. However, large-scale determination of full-length HIV-1 nucleotide sequences, which is the method used in recombinant genome studies, is not practical for molecular epidemiological investigation. Thus, parallel phylogenetic analysis of HIV-1 *env* and

gag genes for subtype determination was proposed to be a protocol of choice for an HIV-1 subtype or recombinant strain surveillance program.

Four recombinant HIV-1 isolates were identified in this study from three regions of Thailand, northern, northeastern, and southern regions. Interestingly, all recombinant HIV-1 isolates were from provinces that share their border with neighboring countries. Moreover, the recombination breakpoint could be identified in the *gag* gene in one recombinant isolate from Nakhorn Si Thammarat Province in the south. The recombinant breakpoint of the isolate with the recombinant *gag* gene was identified at nucleotide sequence position 380 bp which was in the p24 region and the breakpoint was supported by the phylogenetic tree for the corresponding position.

During the years 1995-2000, genetic analysis of HIV-1 CRF01_AE in Thailand was mainly done on strains isolated from Bangkok and Chiang Mai in the north while the epidemiological patterns in the other epidemic areas, including border areas, have not been reported. The HIV-1 CRF01_AE isolated from samples collected in 2002 from 20 provinces in 3 major epidemics including the border areas of Thailand-Loa PDR, Thailand-Vietnam, Thailand-Myanmar, Thailand-Cambodia, and coastal provinces were studied. Phylogenetic trees of CRF01_AE isolates demonstrated that there was no geographic distinction among HIV-1 CRF01_AE isolates in Thailand. The clustering pattern found in the phylogenetic tree for each region were supported by a signature sequence pattern and N-linked glycosylation. The less genetic diversity in the southern CRF01_AE isolates than the northern and northeastern isolates was confirmed by the number of variable positions. Signature sequence pattern analysis demonstrated the GPGQ V3 loop tip was a predominant pattern in HIV-1 isolated from all geographic regions. The differences in both genetic diversity and genetic patterns of CRF01_AE circulated in the south indicates the possibility the HIV-1 epidemic in the south had its own pattern of geographic introduction and distribution.

It was reported that some of HIV-1 CRF01_AE isolates in China spreaded from Thailand to Cambodia to Vietnam and then to China through sexual contact. They were thought to be connected to sexual networks and the HIV-1 epidemic in these locations (Entz *et al*, 2000). These reports may explain the findings in this study that nothern and northeastern isolates form one distinct HIV-1 cluster, which indicates the distribution of HIV-1 CRF01_AE among foreign workers, internal migrants and commercial sex workers along Thailand-Myanmar-Vietnam borders, while southern isolates may be from HIV-1 distribution among fishermen and the population in coastal provinces. The migration of workers or travellers and the link to commercial sex worker contacts may play an important role in the HIV-1 epidemic in Thailand and neighboring countries. Therefore, a better understanding of the social, behavioral and demographic factors associated with HIV-1 transmission among internal migrating workers, which could not be done in this study, will provide insight into the HIV-1 epidemic in the rural area.

Molecular epidemiological data from this study may be used to design surveillance methods and target prevention efforts. The increasing number of recombinant strains of HIV-1 in border and coastal areas suggests that mobile population, such as migrants or foreign workers, and fishermen, play a special role in the HIV-1 epidemic. Moreover, the HIV-1 epidemic in Southeast Asia was reported to be centered in Thailand and becoming more widespread, as well as more complex in terms of the genetic diversity of the circulating strains (Takebe *et al*, 2003). The molecular epidemiology of circulating strains

of HIV-1 in Thailand from this study provide information on new antigenic circulating strains, which may complicate the development of an effective vaccine. This study gives insight into the epidemiology of virus strains responsible for the HIV-1 epidemic in Thailand.

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REFERENCES

Entz AT, Ruffolo VP, Chinveschakitvanich V, Soskolne V, van Griensven GJ. HIV-1 prevalence, HIV-1 subtypes and risk factors among fishermen in the Gulf of Thailand and the Andaman Sea. *AIDS* 2000; 14: 1027-34.

Felsenstein J. PHYLIP. Phylogenetic Inference Package. In. 3.5c ed. Seattle: Department of Genetics, University of Washington, 1993.

Kalish ML, Luo CC, Weniger BG, *et al*. Early HIV type 1 strains in Thailand were not responsible for the current epidemic. *AIDS Res Hum Retroviruses* 1994; 10: 1573-5.

Kuiken C, Thakallapalli R, Esklid A, de Ronde A. Genetic analysis reveals epidemiologic patterns in the spread of human immunodeficiency virus. *Am J Epidemiol* 2000; 152: 814-22.

Limsuwan A, Kanapa S, Siristonapun Y. Acquired immune deficiency syndrome in Thailand. A report of two cases. *J Med Assoc Thai* 1986; 69: 164-9.

Lole KS, Bollinger RC, Paranjape RS, *et al*. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999 Jan; 73: 152-60.

Magiorkinis G, Paraskevis D, Magiorkinis E, Vandamme AM, Hatzakis A. Reanalysis of the HIV-1 circulating recombinant form A/E (CRF01_AE): evidence of A/E/G recombination. *J Acquir Immune Defic Syndr* 2002; 30: 124-9.

Mansky LM. Retrovirus mutation rates and their role in genetic variation. *J Gen Virol* 1998; 79 (Pt 6): 1337-45.

Martin DP, Posada D, Crandall KA, Williamson C. A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. *AIDS Res Hum Retroviruses* 2005; 21: 98-102.

Nerurkar VR, Nguyen HT, Dashwood WM, *et al*. HIV type 1 subtype E in commercial sex workers and injection drug users in southern Vietnam. *AIDS Res Hum Retroviruses* 1996; 12: 841-3.

Ou CY, Takebe Y, Luo CC, *et al*. Wide distribution of two subtypes of HIV-1 in Thailand. *AIDS Res Hum Retroviruses* 1992; 8: 1471-2.

Ou CY, Takebe Y, Weniger BG, Luo CC, *et al*. Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. *Lancet* 1993; 341: 1171-4.

Phanuphak P, Locharernkul C, Panmuong W, Wilde H. A report of three cases of AIDS in Thailand. *Asian Pac J Allergy Immunol* 1985; 3: 195-9.

Ramos A, Nguyen L, Hu DJ, *et al*. New HIV type 1 CRF01_AE/B recombinants displaying unique distribution of breakpoints from incident infections among injecting drug users in Thailand. *AIDS Res Hum Retroviruses* 2003; 19: 667-74.

Shepherd BE, Rossini AJ, Soto RJ, De Rivera IL, Mullins JI. Sampling designs for HIV molecular epidemiology with application to Honduras. *AIDS Res Hum Retroviruses* 2005; 21: 907-14.

Sirivichayakul S, Chantratita W, Sutthent R, Ruxrungtham K, Phanuphak P, Oelrichs RB. Survey of reverse transcriptase from the heterosexual epidemic of human immunodeficiency virus type 1 CRF01_AE in Thailand from 1990 to 2000. *AIDS Res Hum Retroviruses* 2001; 17: 1077-81.

Smith DG. Thailand: AIDS crisis looms. *Lancet* 1990; 335: 781-2.

Subbarao S, Limpakarnjanarat K, Mastro TD, *et al*. HIV type 1 in Thailand, 1994-1995: persistence of two subtypes with low genetic diversity. *AIDS Res Hum Retroviruses* 1998; 14:

319-27.

Takebe Y, Motomura K, Tatsumi M, Lwin HH, Zaw M, Kusagawa S. High prevalence of diverse forms of HIV-1 intersubtype recombinants in Central Myanmar: geographical hot spot of extensive recombination. *AIDS* 2003; 17: 2077-87.

Tee KK, Pon CK, Kamarulzaman A, Ng KP. Emergence of HIV-1 CRF01_AE/B unique recombinant forms in Kuala Lumpur, Malaysia. *AIDS* 2005; 19: 119-26.

Thompson JD, Gibson TJ, Plewniak F, *et al.* The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; 25: 4876-82.

Tovanabutra S, Watanaveeradej V, Viputtikul K, *et al*. A new circulating recombinant form, CRF15_01B, reinforces the linkage between IDU and heterosexual epidemics in Thailand. *AIDS Res Hum Retroviruses* 2003; 19: 561-7.

Traisupa A, Wongba C, Taylor DN. AIDS and prevalence of antibody to human immunodeficiency virus (HIV) in high risk groups in Thailand. *Genitourin Med* 1987; 63: 106-8.

Viputtijul K, de Souza M, Trichavaroj R, *et al*. Heterosexually acquired CRF01_AE/B recombinant HIV type 1 found in Thailand. *AIDS Res Hum Retroviruses* 2002; 18: 1235-7.

Wangroongsarb Y, Weniger BG, Wasi C, *et al*. Prevalence of HTLV-III/LAV antibody in selected populations in Thailand. *Southeast Asian J Trop Med Public Health* 1985; 16: 517-20.

Watanaveeradej V, Benenson MW, Souza MD, *et al.* Molecular epidemiology of HIV type 1 in preparation for a phase III prime-boost vaccine trial in Thailand and a new approach to HIV type 1 genotyping. *AIDS Res Hum Retroviruses* 2006; 22: 801-7.