

THE EPIDEMIOLOGY OF HIV-1 SUBTYPES IN INFECTED PATIENTS FROM NORTHEASTERN THAILAND

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Abstract. In total, 117 HIV-1 infected patients from several provinces in Northeastern Thailand were analysed. All blood samples collected from individuals were confirmed by EIA and Western blot and partially by HIV-1 *gag*-, *pol*- and *env*-PCR. By serotyping with a V3-peptide ELISA, 108 (92.3%) of the sera samples belonged to subtype E, 9 (7.7%) were subtype B. For 10 Thai HIV-1 infections, the serotype and genotype were determined. The genotype was determined by phylogenetic analysis of directly sequenced PCR amplicons, 8 were subtype E, 2 subtype B. For these patients the serotype did correlate with the genotype. Tracing back the origin of Thai patients, it seems that most were infected within early years of the epidemic and the Thai subtype B infected patients have been imported directly from foreign countries via sexual contact. The findings suggest there are two distinct subtypes in Thailand with the majority being subtype E. The relatively high prevalence of subtype B in Northeastern Thailand may be due to the increasing intermix of the two strains (subtypes E and B) and the migration for employment from foreign countries. This may lead to public health concerns regarding surveillance of HIV-1 subtypes and the regulation of potentially infected workers returning from abroad to the country.

INTRODUCTION

The global HIV pandemic is made up of multiple regional and focal epidemics. Different viral variants have spread to human groups because of distinct risk factors (Janssens and UNAIDS). In the United States and Europe, HIV is predominant among homosexuals and intravenous drug users (IDUs); in Africa, heterosexual transmission is predominant. In 2000, the World Health Organization estimated that 5.3 million people were living with HIV/AIDS in South and Southeast Asia (WHO, 2000a; UNAIDS, 2000). While this number made up 23% of the global pandemic, it was estimated that 6% of all AIDS cases occurred in this region. In Thailand, like in most countries of Asia, a rapid increase of HIV-1 infection in the heterosexual population has been observed since 1989 (Weniger *et al*, 1991) and the geographic distribution of the epidemic is broad, involving multiple provinces and risk

groups. Previous serosurveys showed a significant HIV-1 prevalence in IDUs, sex workers, prisoners, STD clinic attenders and blood donors (Praphan, 1989; Ungchusak *et al*, 1989, 1991; Choopanya *et al*, 1991; Nopkesorn *et al*, 1991; Sriraprepasiri *et al*, 1991; Taywasitap *et al*, 1991; Werasit *et al*, 1991). The HIV-1 infection has recently spread rapidly to the general population, mothers and children (The Global Orphan Project, MOPH). By mid 2002, there were an estimated 1.2 million people with HIV-1 infection in Thailand, about 1.9% of the total population of 63 million (Epidemiology Division, MOPH, Bangkok, Thailand).

HIV-1 strains in different geographic regions are highly diverse and have been classified into several distinct genetic groups (M, N, and O) and subtypes from A to K within the M group (Myers *et al*, 1992, 1993; Leitner, 1996; Robertson *et al*, 1999; McCutchan *et al*, 2001). Recombination between subtypes frequently occurs and some recombined HIV-strains are of epidemiological relevance (Peeters, 2000). Such global variation presents a potential problem for the public health strategies of prevention and control of the spread of AIDS. Two major subtypes of HIV-1 were iden-

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tified in infected individuals in Thailand (McCutchan *et al*, 1992; Ou *et al*, 1993). These two genotypes may have been introduced into Thailand by different modes of transmission: heterosexual (subtype E, now called CRF01_AE) versus parenteral (subtype B). The identification of HIV-1 subtypes from patients in Thailand has been described previously (McCutchan *et al*, 1992; Ou *et al*, 1993; Ichimura *et al*, 1994; Kalish *et al*, 1995; Wasi *et al*, 1995; Yu *et al*, 1995; Limpakarnjanarat *et al*, 1998; Louisirirotchanakul *et al*, 1998; Subbarao *et al*, 1998). Almost all the early studies were relatively small with respect to subject numbers and were located in the central and northern parts of Thailand. The subsequent spread of HIV-1 has been observed in the heterosexual population in Khon Kaen and in neighboring provinces of Northeastern Thailand. Northeastern Thailand, the largest region in the country, contains two-thirds of the total Thai population and is a major residence for labor workers from abroad. Nearly 200,000 Thais work abroad each year, 80-90% of these are from the Northeastern Thailand (Oversea Employment Administration Office, Ministry of Labour and Social Welfare, Bangkok, Thailand). The national surveys of HIV-1 transmission among the infected northeastern population increased from 2,953 cases in 1989-1993 to 18,412 cases in 1999 and more than 20,000 cases in 2002 (Regional Medical Sciences Center 6, Khon Kaen, MOPH, Thailand). Despite the fact that HIV-1 is becoming more prevalent in Thai people, it has not been analyzed in detail which subtypes are prevalent within this region. The purpose of this study was to examine the HIV-1 subtypes among infected patients in the northeastern region of Thailand.

MATERIAL AND METHODS

Subjects

EDTA peripheral blood samples were collected from a total of 117 HIV-1 seropositive patients, 98 males (mean age 28.8 ± 2.8) and 19 females (mean age 24.8 ± 1.3) in an internal medicine clinic at Srinagarind Hospital and Regional Medical Sciences Center, Khon Kaen Province. The likely mode of transmission of these cases was heterosexual, except in 4 males, who were

IDU. After giving informed consent, the men and women were interviewed separately and asked about demographic characteristics; possible modes of HIV acquisition, history of sexual behavior and occupation. Whole blood samples were tested for serotypes using the V3-loop peptide ELISA (Procyon Biopharma Inc, Canada) and transported as dried blood filter spots to the Robert Koch-Institute in Berlin for HIV-1 genotyping and some samples were sent for serotyping using peptide-ELISA (Boehringer). The HIV-infections were confirmed by EIA and Western blot and partially by HIV-1 *gag*-, *pol*-, and *env*-PCR. All were PCR-positive for the *env*-gp41 region.

DNA extraction

DNA from the dried blood filter spots was extracted using the Qiamp Blood kit (QIAGEN GmbH, Germany).

Polymerase chain reaction (PCR)

env gp41/PCR: All samples were screened with the diagnostic *env* gp41/PCR using the primer SK68I established in the laboratory. The PCR product was detected by Southern blotting using peroxidase-labeled probes and the ECL-detection System (Schweiger *et al*, 1996).

env C2V5 PCR: To determine the genotype of the HIV-infections the C2V5 region of the gp120 was analysed. The outer and inner primer pairs used in the nested PCR were based on the nucleotide sequence of the subtype alignments of the *env*C2V5

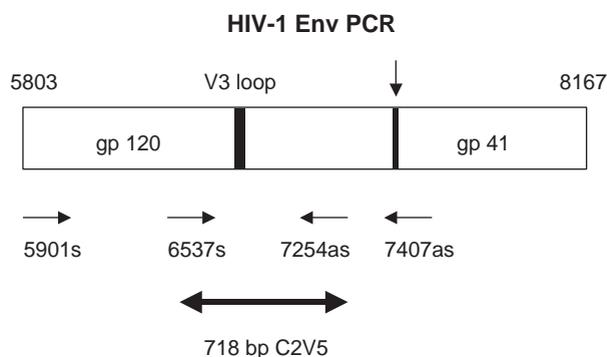


Fig 1—Schematic diagram representing HIV-1 genome with the locations and directions of amplification primers.

region of HIV-1 (Fig 1). The nucleotide sequence of the primers and the locations in the HIV-1 genome are shown in Table 1. The oligonucleotides were synthesized by TibMolBiol (Berlin, Germany).

The envelope *env*C2V5 domain of proviral HIV-1 DNA of infected individuals was amplified by a nested polymerase chain reaction (PCR). The primer pair used in the first amplification was *env*5901s and *env*7407as. After pre-denaturation for 2 minutes at 94°C, a 2 step PCR profile was run for 30 cycles (96°C for 30 seconds, 55°C for 2.5 minutes), followed by a final elongation step of 10 minutes at 68°C. Combi-Polymerase with proofreading activity was used for the PCR (InviTek, Berlin Germany). Two microliters of the outer PCR product was amplified with the inner primer pair *env*6537s and *env*7254 with annealing at 60°C. The resultant PCR product was purified using the Qiaquick PCR Purification kit (QIAGEN GmbH, Germany).

The purified amplicon was directly sequenced using 5 different sequencing primers and fluorescent dye-labeled terminators (AB/Perkin Elmer; Protocol: ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit with Ampli TaqR DNA Polymerase, FS; P/N 402116 Revision A, August 1995). Nucleotide sequences were edited using the DNA Sequencing Analysis Version 2.1.1

Software Program and the autoassembler on Macintosh Computers. The sequencing primer used is shown in Table 1.

Subtyping

Serotyping by peptide-ELISA. The enzyme immunoassay used in this study has been described previously (Pau *et al*, 1993a,b), employing 14-meric peptides from the crown of the V3 loop specific for Thai A (*env* subtype E: TSITIGPGQVFYRT) and Thai B (*env* subtype B: KSIHLGPGQAWYTT). A non-virally coded aspartic acid (D) was added to the N-terminal end of each peptide, yielding a 15-amino-acid peptide, to improve binding to the plates. For plate preparation, the peptide antigens (0.5 µg/100 µl sonicated in pH 9.6 carbonate buffer) were passively adsorbed (110 µl/well) onto the microtiter plates (Immunlon 4; Dynatech, Chantilly, Virginia, USA) overnight at 4°C. The next day, antigen was aspirated and the plates were blocked with 200 µl/well skimmed milk buffer at 37°C for 1.5 hours (5% non-fat dry milk and 0.3% Tween 20 in pH 7.2 PBS), followed by three washings with pH 7.2 PBS containing 0.05% Tween 20 (PBS-Tw) and drying at 37°C for 1 hour. Antigen coated/blocked plates were stored desiccated at -70°C until use (within 3 months). For peptide-ELISA, diluted serum samples (100 µl/well of optimized

Table 1
The nucleotide sequence of the primers and the locations in the HIV-1 genome.

Name	Sequence 5'-3'	Coordinates
PCR primers		
<i>env</i> 5901s	ATTgTgggTCACAgTCATTATggggTACCT	5901-5931
<i>env</i> 7407as	CATAgTgCTTCCTgCTgCTCCCAAgAACC	7407-7379
<i>env</i> 6537s	AATgTCAgCACAgTACAATgTACAC	6538-6562
<i>env</i> 7254as	CCAATTgTCCCTCATATCTCCTCCTC	7254-7231
CO601as	TTCTCCAATTgTCCCTCATATCTCCTCCTCA	7258-7227
Sequencing primer		
C2V5-1s	CagAAgAAgAggTAATAg	6614-6635
C2V5-1as	GCTACTTCTTCTgCTAgACTgCCA	6626-6603
C2V5-2s	TTAATTgTggAggggAATTTTTCT	6894-6917
C2V5-2as	AgAAAAATCCCCCTCCACAATTA	6917-6894
C2V5-3s	TggCAgggAgTAggACAAGCAATgT	7096-7120
C2V5-3as	ACATTgCTTgTCCTACTCCCTgCCA	7120-7096
EC2V5-s	ATATAgAAAAgCATATTgTgA	6791-6812
EC2V5-as	ATATAgAAAAgCATATTgTgA	973-994

serum dilutions in milk buffer:1:400-1:500) were added to the antigen-coated plates and incubated for 1 hour at 37°C. Bound antibodies were detected with peroxidase conjugated goat anti-human IgG (H+L) diluted in milk buffer and tetramethylbenzidine/H₂O₂ substrate after washing three times with PBS-Tw between each step. Absorbance at 450 nm against 630 nm was measured. A cut-off of 0.3 was used throughout the study, with dual reactions further clarified as monoreactive if one peptide optical density (OD) was three x greater than the other peptide OD.

Genotyping by sequence analysis. The sequences derived from proviral DNAs were analysed for their phylogenetic relatedness to reference sequences of HIV-1 subtypes (Felsenstein 1985, PHYLIP package version 3, 52, DNAdist (Kimura 2 Parameter model).

RESULTS

Blood samples were collected from 117 subjects from Khon Kaen and the neighboring provinces of Northeastern Thailand. There were more men (84%) than women, with average ages between 25-29 years. Most of them were labor workers (92%). The likely mode of HIV-1 transmission of these cases was via heterosexual contact, except in 4 men, who were also IDUs.

The serological determination of the HIV-1 subtype by peptide ELISA on the 117 samples showed 108 (92.3%) as subtype E and 9 (7.7%) as subtype B. Among subjects infected with subtype B, 7 probably acquired HIV-1 infection by sexual transmission (spouses of HIV-1 infected persons) and 2 had a history of IDU. Genetic subtype information on the *env*C2-V5 region was analysed from 20 HIV-1 infected patients and all were C2V5 PCR positive with the expected size of 718 bp (Fig 2). Ten out of these positive C2V5 PCR products were subsequently analysed by direct sequencing. The genotype was determined by phylogenetic analysis of the consensus sequences. Eight belonged to the subtype E clade and 2 to subtype B. When V3 loop peptide ELISA results were compared to HIV-1 genotyping results there was a good correlation between the results (Table 2). All 8 of the subtype E samples

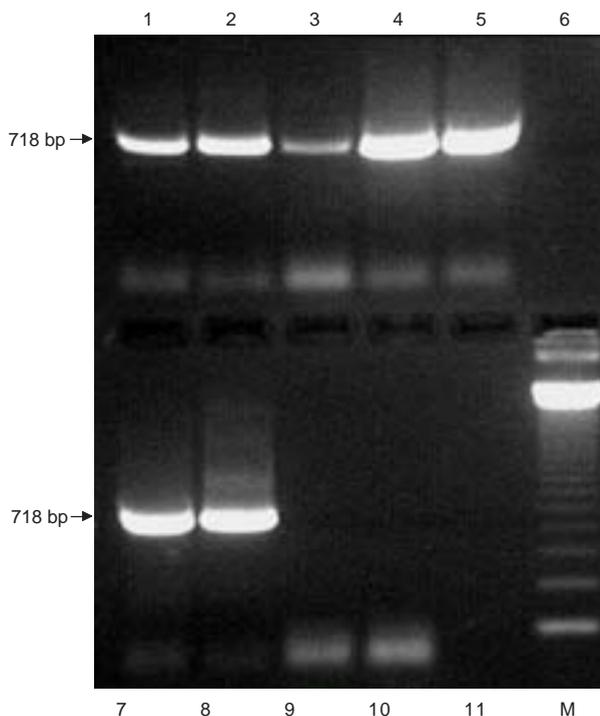


Fig 2—Amplification of *env*C2V5 domain of proviral HIV-1 DNA of infected individuals by nested PCR resulted in positive PCR products of 718 bp (lanes 1-5). DNA pGEMBru (lanes 7-8) and tRNA (lane 9), H₂O (lane 10) are positive and negative controls, respectively. Lane M is *Hind* III DNA markers.

Table 2
Identification of HIV-1 subtype by serotyping with V3-Peptide ELISA compared with C2V5 sequence/Genotype (CLUSTAL).

Method analysis	Specimen no.
C2V5 PCR positive	20
C2V5 Sequence and Genotype (CLUSTAL)	10
V3-Peptide ELISA	10
V3-Peptide ELISA and Genotype (CLUSTAL)	10
Concurrence	10
Discrepancy	0
Not done (genotype)	10
Subtype identification (CLUSTAL)	
A	0
B	2
C	0
D	0
E	8

containing the GPGR tetrapeptide motif in the crown of the V3 loop typical of HIV-1 subtype E in Thailand classified as genotype E were correctly identified by peptide ELISA. Both subtype B specimens were B reactive in the V3 loop peptide ELISA and contained the common GPGQ motif in the V3 loop. Serotyping on the basis of V3 loop reactivity yields reliable results in populations where one subtype is predominant, whereas in populations where several subtypes are cocirculating (M. Hölscher Tanzania and Cheigsong-Popor, personal communication) specificity problems are encountered as well as in populations where the epidemic is older than in Thailand and the spreading viruses are more divergent. Overall, subtype E accounted for 92.3% (108 out of 117) and subtype B accounted for 7.7% (9 out of 117) of HIV-1 infections. There was no significant difference in years of age for persons infected between the HIV-1 subtype E (mean age 28 ± 2.4 years) and subtype B (mean age 29 ± 1.2 years).

DISCUSSION

One of the major characteristics of HIV is its high genetic diversity. Genetic studies reveal the HIV pandemic is made up of multiple subtypes of viruses. It is important to consider the genetic subtype in the development of antiviral vaccines and therapies. HIV sequence data has been used to develop a phylogenetic classification system. An HIV sequence database is available from the Los Alamos National Laboratory in the United States, there are currently 11 genetic subtypes of HIV-1 (Leitner, 1996; McCutchan, 2001). Data available from Southeast Asia show HIV subtype E as the predominant variant with subtype B also circulating in certain population groups (Brown *et al*, 1998).

The molecular epidemiology of HIV in other countries of Southeast Asia has been studied in less detail than in Thailand. Subtype E is wide spread and often is predominant. The subtype has maintained its predominance in Thailand for ten years and appears associated with much of the HIV epidemic throughout the rest of Southeast Asia, with more than a million people infected. The sequencing of the full 10 kilobase genome of subtype E

virus from Thailand revealed that it is a mosaic with multiple crossovers between parental subtype A and parental subtype E, now called CRF01_AE (Carr *et al*, 1996; Peeters, 2000; McCutchan, 2001). The HIV-1 epidemic in Southeast Asia is becoming more complex with respect to HIV-1 diversity. Recent reports include subtype B, 'Thai B', C, D, and CRF01_AE and a B/C recombinant in Southern China. A CRF01_AE/subtype C, CRF01_AE/B recombinant and CRF15_01B have been detected in Thailand (Viputtikul *et al*, 2002; Tovanaabutra *et al* 2001; 2003). These findings are suggestive of more recombinants to come. Intensified monitoring is particularly important in light of the ongoing evaluations of HIV-1 vaccines and antiviral therapies. A significant fraction of various recombinant strains could necessitate a follow-up of the full genome sequencing to evaluate the relative effectiveness of vaccines against the different HIV-1 subtypes. The proportion of HIV subtype E infections among newly infected Bangkok IDUs increased from 3% in 1988-1989 to 44% in 1992-1993 (Wasi *et al*, 1995). The wider epidemic in Thailand is sexually transmitted comprising an estimated 83.2% of the total number of AIDS patients in Thailand, as of December 2000 (Rojanapithayakorn *et al*, 2001). Ongoing surveillance of 21-year-old, male military conscripts shows that serotype E infections make up more than 90% of HIV cases.

Information regarding the distribution of HIV-1 subtypes in patients with AIDS in Northeastern Thailand is scarce and may change over time. Recent studies suggest that the distribution of HIV-1 genetic subtypes can vary significantly from country to country and within population groups, and can be associated with a particular mode of transmission and can change over time (Myers *et al*, 1993; Sharp *et al*, 1994; Weniger *et al*, 1994; WHO, 1994). This variation reflects the dynamic and complex nature of the virus and the epidemic; however, the driving forces behind such variation are not well understood. This study examined the serotype and genetic subtype of HIV-1 in Khon Kaen and neighboring provinces of Northeastern Thailand. The presence of two major distinct HIV-1 genotypes in Thailand provide a unique opportunity to follow the natural history of infection by two viral strains, to find out

potential differences in the progression of disease, pathogenicity, clinical manifestations and transmission efficiency. To facilitate these studies, infections by the genotypes E and B in Thai patients have been differentiated by means of serological tests based on synthetic peptides derived from their respective V3 loops (Pau *et al*, 1993a,b). Our results by serotyping with a V3-peptide ELISA revealed that 108 (92.3%) samples belonged to serotype E, 9 (7.7%) to serotype B. For 10 Thai HIV-1 infections the serotype and genotype were studied. The genotype was determined by the phylogenetic analysis of directly sequenced PCR amplicons. Eight belonged to subtype E, 2 to subtype B. All the serotypes correlated with the genotypes.

It is impossible to find out how and from where the original Thai genotype entered the country, because there is much international travel by both foreigners and Thai people to and from all regions of the world. The International Labor Office (ILO), Geneva, Switzerland, warns of the catastrophic consequences of HIV/AIDS for workers and employers worldwide, projecting a severe decline in the size and quality of the workforce in a number of countries over the next 20 years (WHO, 2000b). The report projects that 15 countries will have approximately 24 million fewer workers in the year 2020 as a result of the AIDS epidemic, including Thailand. Data and trends from sub-Saharan Africa, the worst affected area, and other regions, indicate that effective and large-scale preventive interventions are required to avoid such catastrophes. Employees can also be replaced by importing labor from neighboring countries, at the risk of creating a bigger immigrant sub-population, which is more vulnerable to HIV infection. Since indigenous transmission of HIV in residents of Northeastern Thailand prior to year 1988 has not been well demonstrated (Weniger *et al*, 1991), it is reasonable to assume that these patients progressed to AIDS after an infection on average of 6 years or less. Tracing back the origin of the Thai subtype B infection, it seems that it was imported directly from the foreign countries via sexual contact. This finding is consistent with those found in an earlier report of a smaller sample size and with greater than 95% Thailand genotype E, which was associated with

heterosexual transmission in the country (McCutchan *et al*, 1992; Ou *et al*, 1992, 1993; Limpakarnjanarat *et al*, 1998; Subbarao *et al*, 1998). Recent studies suggest that subtype E can clearly crossover from the heterosexual epidemic to IDU where the substantial reservoir of subtype B in Thailand resides (Subbarao *et al*, 2000; Tovanaabutra *et al*, 2003). These observations confirm that there are two distinct subtypes present in Thailand with the majority being subtype E (CRF01_AE) but the prevalence of subtype B in Northeast Thailand is relatively high due to crossover of these two subtypes and the return of Thais who have worked overseas. This should raise the public health issues of monitoring for the two recombinant strains throughout the country and increased regulations for potentially infected workers returning from abroad. In addition, government and private agencies in Thailand should sustain preventive programs to reduce the spread of HIV-1 infection through education and behavior modification.

ACKNOWLEDGEMENTS

This study was supported financially by Deutscher Akademisches Austauschdienst (DAAD), the Robert Koch-Institute, Berlin, the BMG (Federal Ministry of Health), Germany, Khon Kaen University, and Faculty of Public Health Mahidol University. We also acknowledge all the staff who helped with blood and data collection at Srinagarind Hospital and the Regional Medical Sciences Center, Khon Kaen, MOPH, Thailand.

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