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### Prediction of *gag* and *env* gene on epidemiology of *HIV-1* infection

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#### ABSTRACT

*HIV* was classified into 3 groups: group M, N and O. Group M is an important group for epidemiology. In Thailand, CRF01\_AE and B are the most common subtype. Because of heterogeneity, *HIV* can recombine genes to another subtype and cause a new subtype. Forty-two plasma samples from *HIV*-infected patients were collected at Siriraj Hospital. Specific region of *p17 (gag)* and *C2-V4 (env)* genes of *HIV* were amplified by nested RT-PCR. PCR products were sequenced. Phylogenetic tree was constructed for epidemiological study. Results showed that phylogenetic analysis on *env* gene was found 100% of *HIV* subtype CRF01\_AE (42/42). Whereas on *gag (p17)* gene, 69% and 31% were found in CRF01\_AE and CRF15\_01B subtype, respectively.

**Keywords:** Epidemiology, *HIV*, CRF01\_AE

#### INTRODUCTION

Human Immunodeficiency Virus (*HIV*) is the virus which is caused acquired immunodeficiency syndrome (AIDS). The main immunological features of *HIV* infection lead to a reduction in number of CD4<sup>+</sup> T lymphocyte and inversion of the normal CD4<sup>+</sup>/CD8<sup>+</sup> ratio which is worsen the capability of immune systems. Patients with AIDS have become unusually susceptible to a range of bacterial, viral and parasitic infections which would not normally cause them problems and are likely to develop malignancies such as Kaposi's sarcoma and non-Hodgkin's lymphoma because of the progressive CD4<sup>+</sup> T cell depletion induced by *HIV*. Over a period of months or years, the opportunistic infections become more severe, leading eventually to death [1-5]. More recently, non-AIDS diseases such as cardiovascular disease, co-infection with hepatitis viruses, renal failure are major causes of morbidity and mortality in *HIV* infection [6-8].

Examination of the genetic variability of *HIV-1* in individuals provides important information about the epidemiology of the *HIV* epidemic, the dynamics of viral replication, and likely response rates to treatment regimens. Furthermore, the extent of *HIV* genetic diversity has implications for the development of vaccines as well as strategies to prevent and overcome drug resistance.

On the basis of sequence homology within the *env* gene, *HIV-1* can be divided into 3 groups: M (major), O (outlying), N (new or non-M/non-O) [9]. Recently one putative group, P, was found in Cameroon [10, 11]. The major group (M) has been further subdivided into nine major subtypes/clades, including A-D, F-H, J and K [12-18] which are classified on the basis of 20-50% differences in *env* gene sequences [19]. Furthermore, *HIV* genome

recombination can occur in co-infection with two different subtypes. This combination creates new viral strain which is called circulating recombinant forms (CRFs).

In different geographical regions, different subtypes have been observed. Globally, the predominant HIV-1 variants are subtypes A and C, followed by subtype B and the subtype AE and AG [13, 20-22]. Subtype B has been found mostly in Europe and America. However, in the last decade, at least 25% of newly infected individuals in these regions carry non-B subtype variants. Subtype A and recombinant A/G variants have been identified in west and central Africa. Subtype C has been observed in southern and eastern Africa, India and Nepal [14, 15, 23-32]. The recombinant A/E predominates in South East Asia; Thailand, the Philippines, China and central Africa [14, 33-36]. Because the distributions of the different subtypes are known, they can be used as molecular epidemiological markers to track the course of the HIV-1 pandemic [13, 37].

## EXPERIMENTAL SECTION

### Sample preparation and extraction of HIV-1 RNA

Forty-two peripheral blood from HIV-1 patient patients was provided by Siriraj Hospital. Immediately upon collection (<6 hr), blood in vacutainers (w/anticoagulant ethylenediaminetetraacetic acid, EDTA) was centrifuged at 1500 rpm for 10 min, 1 ml of plasma was removed for HIV RNA quantification. Then, plasma samples were extracted with COBAS® AmpliPrep Total Nucleic Acid Isolation kit (Roche Molecular Diagnostics).

### Reverse transcription of patient HIV-1 RNA into cDNA

To confine our genotype test to HIV-1, we designed 4 primers to amplify relevant regions of the HIV gene.

1. RT-PCR-end (5'-GCTCCCCTGAGGAGTTTACACA-3'),
2. PA3 (5'-GGAATTTTCCTCAGAGCAGACCAG-3'),
3. PA1 (5'-GCTTTACCTTAATCCCTGCATAAA-3') and
4. RT-PCR-start (5'-TTAGTAGGACCTACACCTGTCAACAT-3').

HIV-1 RNA in extracted samples was reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT, Promega, USA). The reaction was carried out by placing 10 µl of RNA into PCR tubes, heated to 70°C (~5 min) to denature its secondary structure, cooled (on ice for ~5 min), mixed with 15 µl of RT-PCR master mix and thermocycled (GeneAmp, USA) for one round of reverse transcription; 42°C for 45 min, 95°C for 5 min, 4°C hold and all ramp times were 1 °C/second. Per-reaction, the master mix consisted of 5.0 µl of 5xMMLV RT buffer, 2.0 µl of 25 mM MgCl<sub>2</sub>, 2.0 µl of 2.5 nM dNTP, 1.0 µl of 10 pmole/µl RT-PCR-end primers, 4.25 µl of distilled water, 0.5 µl of 200 U/µl M-MLV RT enzyme and 0.25 µl of 40U/µl RNase-Inhibitor.

### Amplification of transcribed cDNA

Initially, a standard PCR method amplify *gag* and *env* region of the viral genome followed by a PCR technique. For standard PCR method, 10 µl of the transcribed cDNA was transferred into PCR tubes containing 40 µl of master mix and thermocycled for one cycle at 94°C for 4 min, 35 cycles at (94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min) and one cycle at 72°C for 7 min with a 4°C hold. All ramp times were at 1°C/second. Master mix contained per reaction: 5.0 µl of 10xTaq buffer, 2.0 µl of 25 mM MgCl<sub>2</sub>, 4.0 µl of 2.5 mM dNTP, 1.5 µl of 10 pmole/µl RT-PCR-end primers, 1.5 µl of 10 pmole/µl of PA3 primers, 25.5 µl of distilled water, and 0.5 µl of 5 U/µl of Taq DNA polymerase (Thermo Fisher Scientific, EU).

The expected size of PCR product of 600 and 480 bp were confirmed by 0.7% agarose gel electrophoresis/UV lamp detection with 100 bp DNA markers (Fermentas, Thermo Fisher Scientific, EU). The final PCR product was purified by HiYield™ Gel/PCR Fragments Extraction Kit (RBCBioscience, Taiwan).

### Sequencing of the amplified RT gene

The amplified DNA was sequenced employing 2 primers that we specifically tailored to target expected mutations within the HIV-1 reverse transcriptase region. The primers were of 20-30 bases long providing ideal melting temperature and annealing times for standard PCR reactions. These primers (listed below) were employed by 1<sup>st</sup> BASE DNA Sequencing Services (Malaysia) to sequence the HIV-1 *pol*-RT gene extracted and amplified from patient samples.

1. JH33 (5'- CTGTTIAATGGCAGICTAGC -3'), and
2. P1711 (5'- GAGAGATGGGTGCGAGAGCG -3'),

For sequencing HIV-1 RNA samples. In brief, 4  $\mu$ l of PCR amplified products were added into tube of sequencing master mix which composed of 2  $\mu$ l of RNase free water, 2  $\mu$ l of BigDye sequencing buffer, 4  $\mu$ l of primers, and 4  $\mu$ l of terminator dye. Then, sequencing reaction was performed in Thermal Cycler 9700.

#### **Alignment of DNA sequences and data mining for epidemiology**

With results obtained with the Genetic analyzer, the forward and reverse DNA sequences are compared to the Chromas version 1.45 with the Lasergene software version 5.0 (DNASTAR Inc., WI, USA). DNA sequences obtained with both methods were edited and assembled using a reference sequence on the ClustalX program.

#### **Phylogenetic analysis**

Sequences from the forty-two patients were spliced and codon aligned using TREEVIEW Version 1.6.6. Phylogenetic analyses were performed using software from the Program Manual for the Neighbor-Joining tree and PHYLIP 3.63. Significant bootstrap values from 1000 replicate data sets were generated using ESEQBOOT and ECONSENSE.

### **RESULTS AND DISCUSSION**

#### **Identifying PCR product in the RT gene**

Forty-two blood samples from HIV-1 patients were purified and amplified by PCR. Figure 1 and Figure 2 showed the RT-PCR products of C2-V4 of *env* gene, and P17 of *gag* gene, respectively.

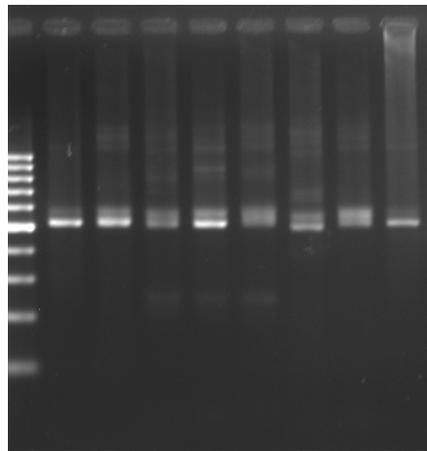


Figure 1. Determination of PCR product; this is a representative gel showing all 600 bp.

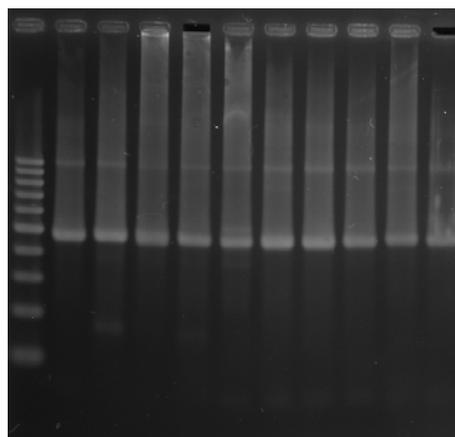


Figure 2. Determination of PCR product; this is a representative gel showing all 480 bp.

#### **Phylogenetic analysis of the env sequences**

Phylogenetic analysis of the forty-two sequences demonstrated that all sequences were consistent with CRF01\_A/E subtype virus. All sequences fell into clusters supported by highly significant bootstrap values (>100%)(Figure 3).



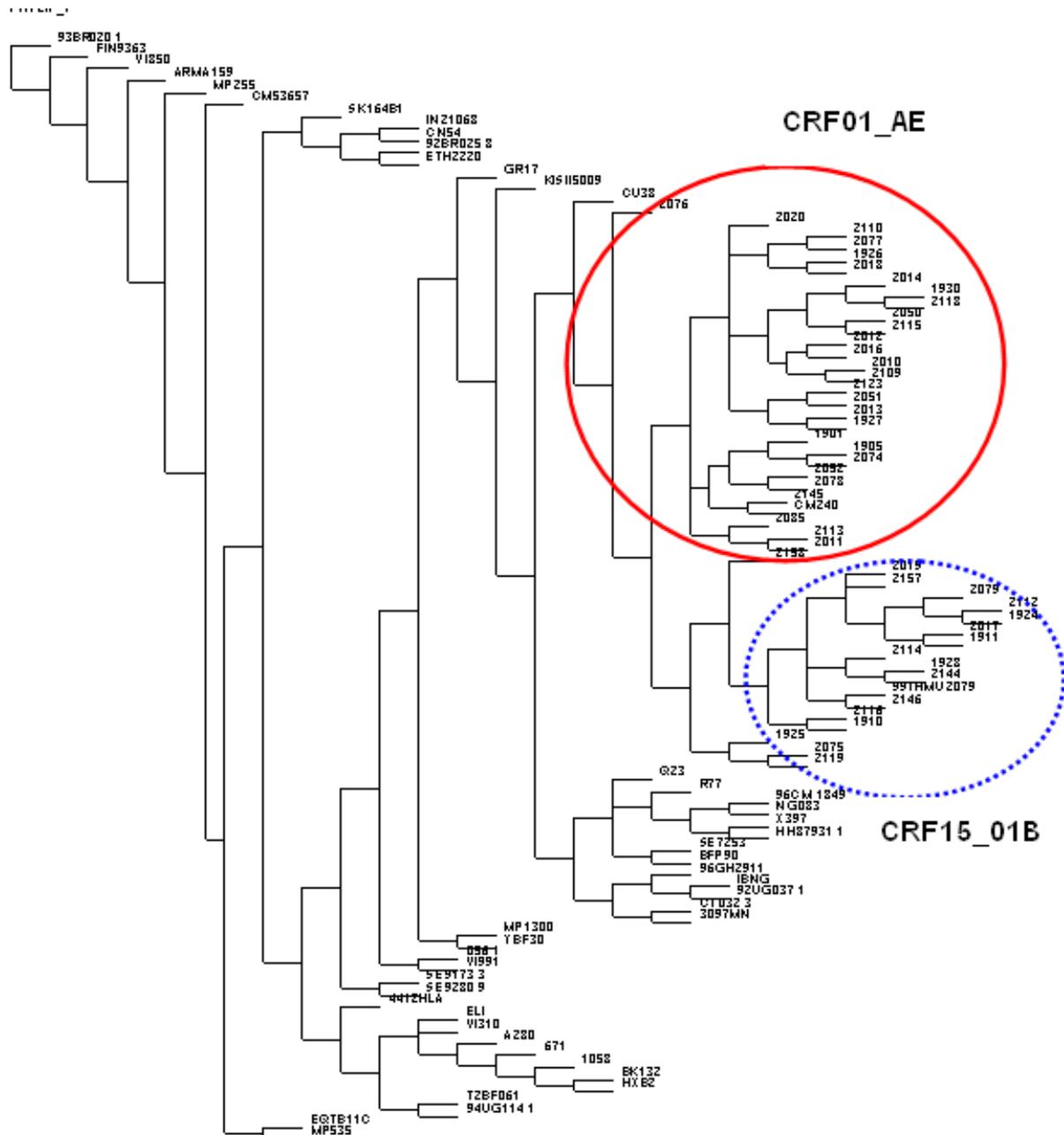


Figure 4. Phylogenetic analysis of the env sequences

## CONCLUSION

From this study showed that the prediction of C2-V4 *env* gene on HIV epidemiology in Thailand was 100% of CRF01\_AE in Bangkok. Our data was slightly different from Wirachsilp P. et al (2007) study. They stated that 95% of CRF01\_AE was found in Bangkok. Furthermore, our study showed that 4% of HIV subtype B was found. However, the phylogenetic tree by using *gag* gene demonstrated that 69% of HIV-1 CRF01\_AE and 31% of HIV-1 CRF15\_01B were found. Sequences fell into thirteen clusters supported by highly significant bootstrap values. The large number of different clusters and the number of unclustered sequences indicate that the population surveyed is probably representative of the overall patterns in Thai population. Combined, these observations suggest that the population sampled is not biased either by multiple transmissions by single individuals, or by preferential transmission of a particular virus with some advantage in transmission or fitness.

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