Sequence Note

Characterization of Mutations in HIV Type 1 Isolates from 144 Cambodian Recently Infected Patients and Pregnant Women Naive to Antiretroviral Drugs

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ABSTRACT

A baseline study has been conducted to determine the polymorphism of reverse transcriptase, protease, and envelope genes of HIV-1 isolates from 146 antiretroviral drug-naive Cambodian patients including 22 seroconverters and 124 pregnant women having been diagnosed HIV positive for less than 1 year. Amplification of at least one gene was successful for 144 isolates. All three genes were obtained for 136 isolates. Subtyping showed that CRF01_AE was predominant (130 cases). According to the ANRS September 2004 list, polymorphism substitutions (>50% versus the subtype B consensus) of CRF01_AE at drug resistance positions were observed only in protease: I13V (81%), E35D (87%), M36I (100%), R41K (96%), and H69K (100%). Two strains bore one major resistance mutation to PIs: M46I and N88D. Five other strains carried drug resistance mutations to RTIs: K70R (one strain), V75M (three strains), and K101E (one strain). Of the isolates 4.9% had drug resistance mutations to antiretroviral drugs.
and C2V3 envelope (env) and to describe the prevalence of mutations involved in resistance to ARVs [nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs)] among untreated patients before the widespread use of ARVs in Cambodia supported by the World Health Organization (WHO) 3 by 5 initiative [http://www.who.int/3by5/en/countries.pdf].

From February 2003 to December 2004, two groups of consecutive patients with no history of antiretroviral therapy gave their informed consent to participate in our study approved by the National Ethical Committee of Cambodia. The first group was repeat consultants to the voluntary counseling and testing center of the Institut Pasteur du Cambodge, with a known date of seroconversion of less than 1 year. The second group was composed of pregnant women attending prenatal clinics at four sites (Calmette Hospital and The National Maternal and Child Health Center in Phnom Penh and the Serey Sophon and Poipet health centers in Beantey Mean Chey province) among the six centers in Cambodia, which applied the PMTCT program in 2003. Pregnant women were accepted in the study if they were tested HIV seropositive for the first time during the previous year and if they declared they were naive to ARVs. In total, 146 patients participated (22 from group 1 and 124 from group 2).

Blood was collected in EDTA tubes. Plasma was separated and stored at $-80^\circ$C before being used for further studies. HIV-1 serology was confirmed positive at the Institut Pasteur du Cambodge with ELISA Genscreen HIV1/2 (Bio-Rad, Marnes la Coquette, France), MEIA (Abbott AXSYM System, Wiesbaden, Germany), and Western blot assay (HIV Blot 2.2, Genelabs Diagnostics Pte Ltd, Singapore). Viral RNA was extracted from plasma using QIAamp Viral RNA

![Graph](image_url)

**FIG. 1.** Amino acid substitutions in the reverse transcriptase (rt) of HIV-1 strains from 144 Cambodians vs. B consensus sequence. The bold boxed columns indicate the positions of resistance mutation according to the ANRS algorithm September 2004 and gray highlights indicate significant (>50%) polymorphism positions. Position 1–5 amino acids are not shown as they correspond to the inner primer of rt. Amino acids 6–11, 244–250, 245–250, 247–250, and 249–250 were missing for one, one, two, and one sequences, respectively.
mini kit (Qiagen, Hilden, Germany), reverse transcribed to cDNA and amplified for prot, rt, and env genes using the Agence Nationale de Recherche sur le Sida (ANRS) consensus sets of primers. Alternative primers were used when amplification with the ANRS primers was unsuccessful. The alternative primer sequences were as follows (positions in parentheses are indicated according to HXB2 sequence accession number K03455): PI-1685 5'-GGGATTTCTCTCA-GAGCAGACCAG (2125–2149) and PI-2209 5'-TCTTCT- GTCAATGCGACAGTGGT (2610–2635) as outer primers, and PI-1685 and PI-2172 5'-CAATCTGCTGTTT- TAAATGGTACG (2572–2593) as inner primers. The alternative prot primer sequences were as follows (positions in parentheses are indicated according to HXB2 sequence accession number K03455): PI-1685 5'-GGAATTTTCCTCA-GAGCAGACCAG (2125–2149) and PI-2209 5'-TCTTCT- GTCAATGCGACAGTGGT (2610–2635) as outer primers, and PI-1685 and PI-2172 5'-CAATCTGCTGTTT- TAAATGGTACG (2572–2593) as inner primers. The fragments obtained were sequenced on both strands using Genome Express company (Meylan, France). Unverified sequences and chromatograms sent by the company were verified, analyzed, interpreted, and aligned. ClustalX 1.81 software was used for alignment sequences with subtype reference sequences set from the HIV sequence Los Alamos Database (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html) and for phylogenetic analysis genotypes using a nucleotide-distance matrix and the bootstrap neighbor-joining method. The presence of drug resistance-associated mutations (DRM) in the prot and rt was detected, and drug susceptibility based on subtype B was predicted from the ANRS algorithm updated in September 2004 (http://www.hivfrenchresistance.org/lab2004.html).

HIV-1 infection was serologically confirmed for all the 146 patients using Western blot assay. However, the amplifications of the three targeted genes were unsuccessful for two pregnant women. Sequences of all the three genes were obtained for 136 individuals, whereas env and/or prot sequences were missing for eight patients. The failure to amplify could be explained by unusual polymorphisms in the targeted regions inducing a primer template mismatch or by a low viral load (data not available in this study), rather than sample degradation. The phylogenetic analysis performed on the three genes for 136 patients revealed that CRF01_AE was predominant (130 patients, 95.5%) followed by subtype B, CRF15_01B, and unknown recombinant forms (two patients, 1.5% each). Phylogenic analysis performed with an uncompleted set of sequences for eight patients showed only CRF01_AE sequences. This result confirms that this subtype is predominant in Cambodia, and likewise in neighboring countries of Southeast Asia.4,5,11

The amino acid substitutions of the Rt are described in Fig. 1. When compared with subtype B consensus, substitutions of amino acids in CRF01_AE strains occurred significantly (>50%) outside ANRS DRM sites, at positions 6, 11, 35, 39, 122, 123, 173, 174, 177, 178, 207, 211, 214, 238, and 245. All these positions of mutations, except positions 6, 11, and 238,
were shared with other consensus subtypes and none of these consensus mutations occurred in an important site of the RT protein (Fig. 2). The deduced CRF01_AE Cambodian natural consensus (>50%) was in agreement with the CRF01_AE consensus observed among Southeast Asian untreated patients and described in previous studies. Differences between these consensus were very few. The Thai and the Singaporean consensus differed from the Cambodian and the Vietnamese consensus at position 43. The Singaporean consensus had no mutation at position 178 while only the Vietnamese consensus had two mutations at positions 162 and 230 (Fig. 2). These mutations could constitute hallmarks of the CRF01-AE strains predominant in these countries. In our study, mutation K43E occurred for 56 (40%) of the 140 CRF01_AE strains (Fig. 1) and the codon 43 is one of the 14 codons that are associated with polymerase activity and substrate binding (Fig. 2). Some other mutations were found at low frequencies outside DRM sites (Fig. 1). According to several studies looking for mutations selected by ARVs among CRF01_AE strains compared to subtype B strains, none of these sites has been shown to be a candidate site for DRM in CRF01_AE strains. Recently, the mutation K238R, especially when accompanied with V106A or V108I, has been associated with resistance to nevirapine (NVP) and is not included in the ANRS and International Aids Society (IAS)-USA lists. The Southeast Asian CRF01_AE consensus carried the K238R mutation (Fig. 2). One of our strains carried the mutations V108I and K238R. Susceptibility of such strains should be phenotypically investigated to estimate the importance of these mutations. According to the ANRS list, DRM to NNRTIs or NRTIs were rare and were observed only among five HIV-1 strains from pregnant women. One CRF01_AE strain carried the mutation K70R, an NAM, which is known to

![FIG. 3. Amino acid substitutions in the protease of HIV-1 strains from 139 Cambodians vs. B consensus sequence. The bold boxed columns indicate the positions of resistance mutation according to the ANRS algorithm September 2004 and significant polymorphism positions are indicated in gray columns.](image1)

![FIG. 4. Comparison of protease consensus (>50%) sequences of different subtypes from untreated patients. PCONS_CAM is the AE consensus sequence from 135 isolates of our present study; PCONS_TH is the AE consensus sequences of 14 isolates from Thailand; PCONS_VN is the AE consensus sequences of 172 isolates from Vietnam; PCONS_SG is the AE consensus sequences of 35 isolates from Singapore. The rest of the consensus is from sequences of M group (A–K) in the Los Alamos National Laboratory database (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REFAlign.html). Residues identical to those of the PCONS_B are indicated by dots.](image2)
be associated with cross-resistance to NRTIs; three CRF01_AE strains carried the mutation V75M, associated with resistance to stavudine (d4T), and one B strain carried the mutation K101E, associated with resistance to the NNRTIs efavirenz (EFV) and nevirapine (NVP).

When compared with subtype B consensus, mutations of CRF01_AE strains at ANRS DRM sites occurred significantly (>50%) at positions 13, 35, 36, 41, and 69, while only one occurred significantly outside DRM sites at position 89. Globally, when considering all observed substitutions whatever their frequencies, amino acid substitutions occurred in 41 (43%) of the 99 positions among CRF01_AE strains and 18 (18%) were situated at ANRS DRM positions 10, 13, 20, 24, 33, 35, 36, 41, 45, 46, 62, 63, 69, 74, 77, 82, 88, and 91. The diversity was the highest at position 63 (Fig. 3). The deduced CRF01_AE Cambodian natural consensus (>50%) was in agreement with the CRF01_AE consensus observed among isolates from Southeast Asian untreated patients (Fig. 4). Only the Vietnamese consensus showed one difference at position 16. None of these Cambodian CRF01_AE consensus mutations occurred in a significant site of the protein, except the E35D substitution (located in the active site of the enzyme). The mutation M36I was carried by all non-B consensus while mutations R41K, H69K, and L89M were observed in most of them (Fig. 4). All our 139 protease sequences had at least one ANRS DRM (minor mutations) but only two strains, belonging to subtype CRF01_AE and from pregnant women, were carrying one major DRM each: M46I associated with resistance to indinavir and ritonavir, and N88D associated with resistance to saquinavir. In addition, we detected two strains with the treatment-related mutations T74A/S, which have an original genotype (e.g., consensus rt and prot genes, rt carrying the mutation K43E, prot carrying the mutation T74A/S, etc.) in order to appreciate their role in the resistance or the susceptibility of such HIV-1 strains to RTIs and PIs, respectively.

SEQUENCE DATA

GenBank accession numbers for the sequences reported in the study are DQ013570 to DQ013573 for the partial reverse transcriptase gene, DQ013514 to DQ013652 for the protease gene, and DQ013653 to DQ013791 for C2V3 envelope gene.

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