Arch Virol (2007) 152: 1799–1805
DOI 10.1007/s00705-007-1019-9
Printed in The Netherlands

Archives of Virology

Polymorphisms and drug resistance analysis of HIV-1 CRF01_AE strains circulating in Fujian Province, China

J. Liu[1,2], **J. Yue**[1], **S. Wu**[2], **Y. Yan**[2]

[1] Department of Clinical Laboratory, Shanghai Pulmonary Hospital, Shanghai, China
[2] Fujian Center for Disease Control and Prevention, Fuzhou, Fujian, China

Received 2 February 2007; Accepted 31 May 2007; Published online 6 July 2007

Summary

Background. The database of genotypic drug resistance mutations in HIV-1 subtype B circulating in developed industrial countries has been well established; however, little is known regarding the prevalence of genotypic resistance patterns in patients harboring non-subtype-B HIV-1 variants in most Asian countries.

Objective. To characterize the polymorphisms and emergence of drug-resistance mutations, resistance to antiretroviral drugs in naïve and pretreated patients infected with HIV-1 CRF01_AE isolates in Fujian province, China.

Methods. HIV-1 *pol* amplicons from 52 pre- and 14 post-treatment samples were obtained by reverse transcription-polymerase chain reaction (RT-PCR) and sequencing. All of the 14 antiretroviral-treated patients were under a fixed regimen of stavudine (d4T), lamivudine (3TC) and nevirapine (NVP), and they had been on treatment for a mean of 6 months (SD, 4 months).

The sequence data were analyzed using the Bioedit software, and the data regarding drug resistance mutations were obtained using the Stanford software (http://hivdb.stanford.edu).

Results. In comparison with the consensus sequence of B strains, the most common protease polymorphisms in HIV-1 CRF01_AE strains prevailing in Fujian Province, China, were I13V (76.9%), E35D (76.9%), M36I (100%), R41K (98.1%), H69K (90.4%), and L89M (96.2%). Protease mutations between CRF01_AE strains and B′ variants prevailing in China were observed. The proportion of substitutions L63P, A71T/V, V77I and I93L in subtype B′ sequences was considerably higher than in CRF01_AE viruses, while the proportion of L10I, M36I and K20R/I substitutions in subtype B′ sequences was relatively lower than in CRF01_AE strains. A high level of resistance to nucleoside reverse transcriptase inhibitors (NRTIs) (28.6%, 4/14) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (35.7%, 5/14) was found in treatment-experienced patients. High-level resistance to nevirapine (NVP) and lamivudine (3TC) was found in the stavudine/lamivudine/nevirapine (d4T/3TC/NVP) treatment regimen. The overall drug resistance rate was 42.9% (6/14), the resistance rates

Correspondence: Yansheng Yan, Ph.D., 76 Jin Tai Lu, Gu Lou Qu, Fujian Center for Disease Control and Prevention, Fuzhou, Fujian 350001, P.R. China
e-mail: njrmcljf@yahoo.com.cn

to two and to all three drugs under treatment were 14.3% (2/14) and 7.1% (1/14), respectively.

Conclusion. This study is the first report on polymorphisms and emergence of drug-resistance mutations in HIV-1 subtype CRF01_AE prevailing in China. These findings provide useful information on global HIV genetic variability and non-B drug resistance.

Introduction

The initiation of highly active antiretroviral therapy (HAART) has remarkably reduced the morbidity and mortality caused by HIV-1 infection [8]. The emergence of drug resistance variants is the main obstacle to the effectiveness of antiretroviral therapy (ART) [2]. Hence, it is important to evaluate the prevalence rate and mutation patterns of drug resistance in both naive and treated patients.

Resistance to antiretroviral agents often results from mutations within the HIV-1 *pol* gene. Currently, a drug resistance database focused on HIV-1 subtype B has been well established, however, given the increasing genetic heterogeneity of HIV-1 worldwide, it is also necessary to characterize *pol* sequences from a wide spectrum of HIV-1 subtypes occurring among drug-naive populations [9]. Here, we report for the first time the polymorphisms and prevalence of drug resistance mutations in HIV-1 subtype CRF01_AE isolates prevailing in China and provide some insights on the subtype-specific variation and drug resistance spectrum of non-subtype B.

Materials and methods

Specimens

Since 2003, we have been conducting the present study in Fujian Province, China. Our study cohort consisted of 66 individuals infected with HIV-1 subtype AE verified by sequence analysis; these subjects were randomized and included 52 treatment-naive patients and 14 treatment-experienced patients. All patients had given written informed consent, and demographic data was obtained through a personal interview. The subjects were considered treatment-naive if they had never been exposed to antiretroviral drugs and treatment-experienced if they were receiving reverse transcriptase (inhibitors (RTIs) and/or protease inhibitors (PIs) at the time at the time of obtaining the sample.

RNA extraction, reverse transcription-polymerase chain reaction and sequencing

Viral RNA was extracted from 140 µl plasma by using the QIAamp Viral RNA Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA template was then reverse-transcripted to cDNA using a random primer. Nested polymerase chain reaction (PCR) was used to amplify a 1115-bp fragment of the *pol* gene from the cDNA template; this partial *pol* gene comprised the entire PR-encoding regions and the first 230 codons of the RT-encoding sequences. The first-round PCR primers were IBF1 (5′-AAATGATGACAGCATGTCAGGGAGT-3′; HXB_2,1823-1847) and IBR1 (5′-AACTTCTGTATATCATTGACAGT CCA-3′; HXB_2, 3303-3278); The second-round primers were PRB (5′-ACTGAGAGACAGGCTAATTTTTTAG GGA-3′; HXB_2, 2068-2095) and IBR2 (5′-CAAAGGAATG GAGGTTCTTTCTGATG-3′; HXB_2, 3210-3185). The positive PCR products were purified by using a Qiagen Gel Extraction Kit. The HIV-1 reverse transcriptase (RT) and protease (PR) genes were sequenced at Invitrogen (Shanghai, China), with PRB and IBR2 as sequencing primers.

Drug resistance analysis

The HIV-1 RT and PR gene sequences were first edited and assembled using the Bioedit software, and then analyzed for genotypic antiretroviral resistance using the HIVdb program (http://hivdb.stanford.edu). The drug resistance profiles were analyzed based on genotypic and phenotypic interpretations defined by the Stanford HIV RT and Protease Sequence Database. This program identifies primary and secondary resistance mutations at specific codons in the PR and RT regions and determines whether these mutations confer resistance or sensitivity to certain nonnucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), or protease inhibitors (PIs). The HIV drug resistance mutation scoring system established by Prof. Shafer, Stanford University, was used to assess resistance interpretations and to comment on a drug. In this system, the mutation scores for each of the mutations associated with resistance to a particular drug were added to obtain an aggregate score.

Results

Polymorphisms of HIV-1 CRF01_AE prevailing in Fujian, China

A total of 52 treatment-naive and 14 antiretroviral-treated HIV-1-infected individuals were enrolled in this study; all of the subjects were infected with CRF01_AE subtype, verified by subtyping analysis of the *env*, *gag* and *pol* genes. All of the patients

Table 1. Polymorphisms of protease in HIV-1 CRF01_AE isolates in 52 HIV/AIDS treatment-naive patients

Position[a]	Subtype B consensus[b]	Subtype CRF01_AE consensus[b]	Polymorphic amino acids and proportion in CRF01_AE in China (%)
10	L	L	L (82.7), I (13.5), V (1.9), P (1.9)
13	I	V	V (76.9), I (23.1)
14	K	K	K (96.2), R (3.8)
15	I	I	I (94.2), V (5.8)
16	G	G	G (78.8), E (19.2), A (1.9)
19	L	L	L (96.2), I (3.8)
20	K	K	K (78.8), R (11.5), I (9.6)
31	T	T	T (98.1), P (1.9)
35	E	D	D (76.9), E (23.1)
36	M	I	I (100.0)
41	R	K	K (98.1), R (1.9)
43	K	K	K (96.2), R (3.8)
55	K	K	K (88.5), R (11.5)
57	R	R	R (92.3), T (7.7)
63	L	L	L (80.8), P (17.3), V (1.9)
69	H	K	K (90.4), R (5.8), Q (1.9), S (1.9)
70	K	K	K (92.3), R (7.7)
72	I	I	I (92.3), V (7.7)
74	T	T	T (96.2), S (3.8)
82	V	V	V (90.4), I (9.6)
89	L	M	M (96.2), I (1.9), L (1.9)
93	I	I	I (63.5), L (32.7), V (3.8)
99	F	F	F (90.4), L (5.8), I (1.9)

[a] The number shows the amino acid positions in the protease sequence.
[b] The consensus sequences were obtained form the HIV databases (http://www.hiv.lanl.gov).

were local residents of Fujian province; their age ranged from 30 to 50 (mean 34.9 years), with a male: female ratio of 1.3:1. The majority were infected through heterosexual contact (71.2%), followed by IDU (injecting drug user, 10.6%). Seventeen (25.8%) patients were infected abroad, while the other patients were infected locally. All of the 14 antiretroviral-treated patients were under an unchanged regimen of stavudine (d4T), lamivudine (3TC) and nevirapine (NVP), and they had been on treatment for a mean of 6 months (SD, 4 months).

Polymorphisms of the CRF01_AE strains obtained in this study were defined as variation from the subtype B consensus amino acid sequence. The overall spectrum of protease is detailed in Table 1. The most common substitutions were I13V (76.9%), E35D (76.9%), M36I (100%), R41K (98.1%), H69K (90.4%), and L89M (96.2%); the other substitutions with less frequency included L10I (13.5%), G16E (19.2%), K20R/I (21.2%), K55R (11.5%), L63P (17.3%), V82I (9.6%), and I93L (32.7%). In the RT region, the substitutions with proportions >10% were E6D (94.2%), K11T/A (73.1%), V35T (94.2%), T39K (90.4%), K43E (82.7%), K122E (90.4%), D123S/N (96.2%), I135L/T (21.2%), K173I (84.6%), Q174K (96.2%), D177E (96.2%), I178M (32.7%), V179I (13.5%), and S191F (11.5%). The consensus amino acid sequence derived from the 52 treatment-naive patients was identical to the international CRF01_AE consensus at all positions, except at one position, codon 178 at RT region, in which the former was 178I, the same as in the subtype B consensus sequence, while the latter contained the codon 178M (Fig. 1).

A comparison of the sequences of 52 CRF01_AE treatment-naive sequences with those of previously reported [4] subtype B′ variants circulating in China revealed some subtype-specific features in the PR regions. The substitutions L63P, A71T/V, I72V, V77I and I93L existed in most subtype B′

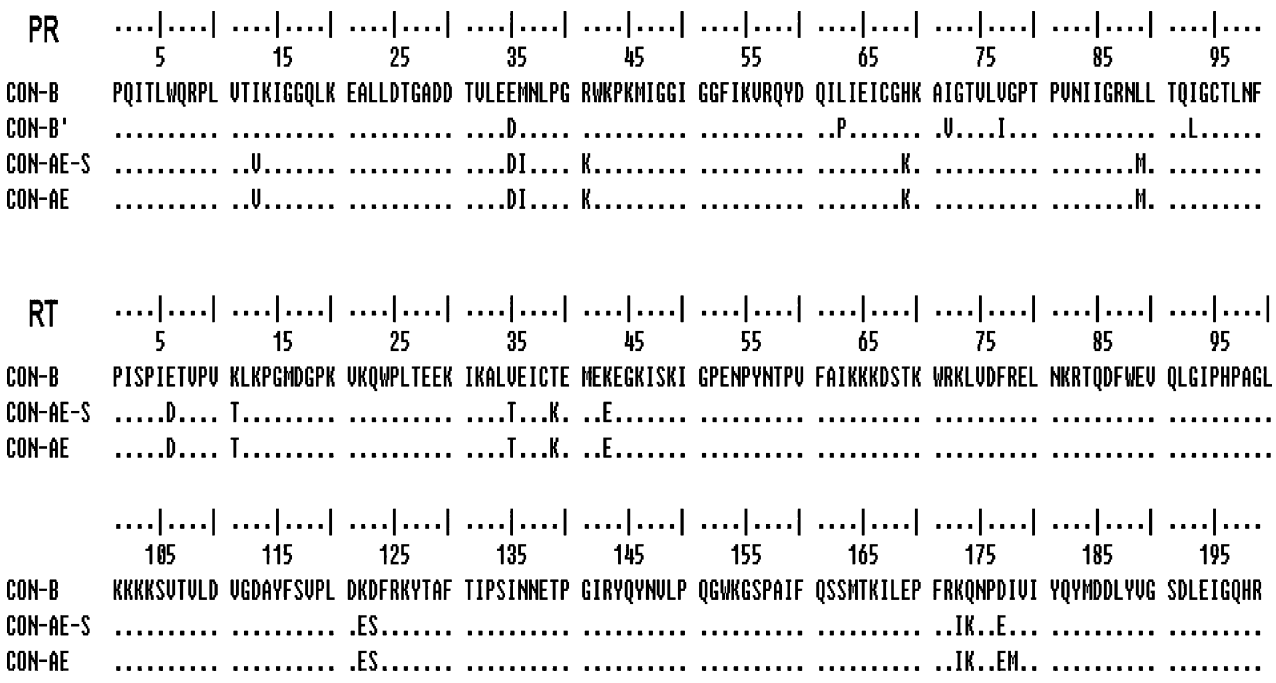

Fig. 1. Alignment comparison of amino acid sequences of protease and RT among subtype B, B′ and CRF01_AE consensus sequences. CON-B and CON-AE are consensus sequences of subtype B and CRF01_AE, respectively, from the HIV databases (http://www.hiv.lanl.gov). CON-B′ is the previously reported [4] consensus sequence for subtype B′ in China. CON-AE-S is the CRF01_AE consensus sequence from this study. In each group, amino acids that are the same as the first sequence are presented a dots

variant sequences but showed very low frequency in CRF01_AE sequences. On the other hand, the substitutions I13V, M36I, R41K, H69K, L89M were found in more than 75% of the CRF01_AE sequences but were observed in only a few or none of subtype B′ sequences. The substitution E35D was present in a high proportion in both subtype B′ and CRF01_AE sequences.

Drug resistance analysis

With regard to the amino acid sequence associated with drug resistance, we noted only one difference, i.e., codon 36 in the PI region (Fig. 1), between the CRF01_AE consensus sequence obtained and the subtype B consensus sequence. This indicated that the drug resistance interpretation system developed by Stanford University for the B clade could be applied to the CRF01_AE clade detected in this study.

In the sequences of CRF01_AE strains from both treatment-naive and treatment-experienced groups, we found a large number of minor mutations, including M36I, I93L, L63P, K20R/I, L10I and V77I. These mutations were also considered to be polymorphisms as mentioned above and did not confer any resistance to PIs. No major mutations associated with resistance to PIs were found in CRF01_AE sequences from any of the patients.

The mutation patterns of resistance to NRTIs and NNRTIs in the drug-naïve and treatment-experienced groups are shown in Table 2a and b. In the treatment-naive group, only 2 samples (4.0%, 2/52) showed mutations associated with resistance to NRTIs or NNRTIs. One sample had NRTI-related mutations A62V and T69N; these mutations only caused potential low-level resistance to drugs.

Among treatment-experienced patients, the resistance levels to one or more NRTIs and NNRTIs were 28.6% (4/14) and 35.7% (5/14), respectively; in the CRF01_AE strains from these patients, the NRTI-related mutations included M184V (7.1%, 1/14), K65R (7.1%,1/14), D67G (7.1%,1/14), K70R (7.1%,1/14), and K70Q (7.1%,1/14), while

Table 2. NRTI and NNRTI mutation patterns in the drug-naïve and drug treatment groups

(a) Resistance patterns in the drug-naïve group

Study subject	Mutation profile (NRTI)	Mutation profile (NNRTI)	NRTIs							NRTIs		
			3TC	ABC	AZT	D4T	DDI	FTC	TDF	DLV	EFV	NVP
FJ16	K70Q	/	S	S	P	S	S	S	S	/	/	/
FJ43	A62V, T69N	/	S	S	S	P	P	S	S	/	/	/

(b) NNRTI resistance patterns in the treatment-experienced group

Study subject	Mutation profile (NRTI)	Mutation profile (NNRTI)	NRTIs							NRTIs		
			3TC	ABC	AZT	D4T	DDI	FTC	TDF	DLV	EFV	NVP
FJ10	/	Y188L	/	/	/	/	/	/	/	L	H	H
FJ14	K70Q	/	S	S	P	S	S	S	S	/	/	/
FJ18	K70R, D67G	K103N	S	S	L	I	S	S	P	H	H	H
FJ33	/	Y181C	/	/	/	/	/	/	/	H	I	H
FJ57	M184V	G190A	H	P	S	S	P	H	S	S	I	H
FJ69	K65R	K103N, Y181C	I	L	S	L	I	I	I	H	H	H

3TC Lamivudine; *ABC* abacavir; *AZT* zidovudine; *D4T* stavudine; *DDI* didanosine; *FTC* emtricitabine; *TDF* tenofovir; *DLV* delavirdine; *EFV* efavirenz; *NVP* nevirapine; *NRTIs* nucleoside reverse transcriptase inhibitors; *NNRTIs* non-nucleoside reverse transcriptase inhibitors; *S*, *P*, *L*, *I* and *H* indicate susceptible, potential low-level, low-level, intermediate-level, and high-level resistant to drugs, respectively.

the NNRTI-related mutations included K103N (14.3%,2/14), Y181C (14.3%,2/14), G190A (7.1%, 1/14) and Y188L (7.1%,1/14). Three (21.4%) samples showed mutation-associated resistance to both NRTIs and NNRTIs. With regard to NRTIs, the resistance level to 3TC, abacavir (ABC), zidovudine (AZT) and emtricitabine (FTC) was 14.3% (2/14) and that to D4T and dideoxyinosine (DDI) was 21.4% (3/14). Among these, a high-level of resistance was observed to 3TC and FTC. Furthermore, high level of resistance was observed to the three NNRTIs, with a rate of 35.7% (5/14).

In the D4T/3TC/NVP treatment regimen, a high level of resistance to NVP and 3TC was observed. The overall drug resistance level was 42.9% (6/14), and the levels of resistance to two and all three drugs were 14.3% (2/14) and 7.1% (1/14), respectively.

Discussion

The current data on effectiveness of antiretroviral therapy and the selection of drug resistance are largely confined to HIV-1 subtype B, the clade that has circulated in North America and Europe. However, subtype B accounts for only ∼10% of the global HIV pandemic. The increasing access to treatment of HIV-1 in the developing countries and increasing non-subtype B infection through travel and migration pose new questions on the susceptibility and response of diverse HIV-1 strains to antiretroviral drugs [6]. An increasing number of in vitro and in vivo studies suggest that the currently available PIs and RTIs are as active against non-subtype-B viruses as they are against subtype B viruses. However, few data are available on the genetic mechanisms of drug resistance in non-subtype B viruses [11]. Hence, it is essential to establish sequence information data on non-subtype B viruses. Here, we report for the first time the polymorphisms and prevalence of drug resistance mutations in subtype CRF01_AE isolates prevailing in Fujian province, southeast China.

In this study, we detected a larger number of polymorphisms in PR of CRF01_AE than in the subtype B consensus sequence. This finding was completely consistent with that previously reported [10] in which many substitutions were considered minor mutations, associated with drug resistance.

It has been reported that PR amino acid changes known to contribute to drug resistance occur as natural polymorphisms in 75% of all HIV-1 strains in patients who never received PIs, and the patterns of these substitutions differs between subtype B and non-B HIV-1 variants [12]. PR substitutions L10I/V/R, K20R/I, M36I, M46L/I, L63P/S/T, A71T/V, V77I, V82A/T/S/F and I93L have been defined as minor (also referred to accessory, secondary and associated) resistance mutations. In our study, the substitution M36I was found in all CRF01_AE strains. This mutation was weakly associated with indinavir, ritonavir and nelfnavir resistance when present with other mutations [5]. Recent in vitro studies showed that in the absence of drugs, the M36I clone replicated more rapidly than the wild-type virus, and K20I and/or M36I improved the replicative capacity of the virus under drug pressure, thereby reducing its susceptibility to saquinavir and indinavir [3]. Thus, our prevalent CRF01_AE strain that harbors M36I may potentiate the relative vitality of virus towards PIs. Other substitutions found as minor mutations were L10I (13.5%), K20R/I (21.2%), L63P (17.3%), I93L (32.7%). The latest report of the RESIST trials showed that H69K was weakly associated with a decreased virological response to Tipranavir [1]. Hence, it can be inferred that the high proportion of H69K (90.4%) in our CRF01_AE strains may has some impact on drug susceptibility.

The positions and proportions of substitutions considered minor mutations were different between our CRF01_AE strains and subtype B′ variants prevailing in China. The proportion of substitutions L63P (90.2%), A71T/V (31.6%), V77I (96.6%) and I93L (91.4%) in subtype B′ [4] sequences was considerably higher than in CRF01_AE viruses, while the proportion of substitutions L10I (3.4%), M36I (0%) K20R/I (0%) in subtype B′ sequences was relatively lower than in CRF01_AE strains.

Comparing the polymorphisms described in this study with those previously reported in CRF01_AE strains found in other region of the world by Kantor et al. [7], some minor discordance were found. In the PR region, the rates of polymorphisms at codon 16, 35, 70 were about 35, 95 and 20%, respectively, in the study by Kantor, while in our study the rates were 21.2, 76.9 and 7.7%, respectively. In Kantor's study, the mutation frequency at codons 45 and 51 varied significantly between CRF01_AE and B strains although the rates were only about 5%. However, the sequences at these two position were highly conserved and present no polymorphism in our study. In the RT region, the mutation frequencies at codons 162 and 178 were higher than 50% in the study by Kantor et al. while in our study the rates were only about 30%. The substitution of RT L74V was not detected in this study. Overall, the polymorphisms of CRF01_AE strains circulating in China were much the same as in the CRF01_AE strains found in other parts of the world.

In summary, this study for the first time characterizes subtype-specific polymorphisms in CRF01_AE strains prevailing in China, and it reveals differences in the proportion and positions of polymorphisms between local CRF01_AE and those in other regions of the world. The prevalence of drug resistance in people receiving ART is at a high level, and some specific resistance patterns have been noted. Further cohort data regarding drug resistance in antiretroviral-treated patients is needed for the evaluation of drug response and evolution of drug resistance mutations in CRF01_AE strains.

Acknowledgment

This work was supported by a grant from Fujian scientific and technological research program (No. 2004YZ01-2).

Appendix

The GenBank accession numbers of the sequences presented in this study are from EF432660 to EF432725.

References

1. Baxter JD, Schapiro JM, Boucher CA, et al. (2006) Genotypic changes in human immunodeficiency virus type 1 protease associated with reduced susceptibility and virologic response to the protease inhibitor Tipranavir. J Virol 80: 10794–10801
2. DeGruttola V, Dix L, D'Aquila R, et al. (2000) The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. Antivir Ther 5: 41–48

3. Holguin A, Sune C, Hamy F, et al. (2006) Natural polymorphisms in the protease gene modulate the replicative capacity of non-B HIV-1 variants in the absence of drug pressure. J Clin Virol 36: 264–271
4. Jiang SL, Xing H, Si XF, et al. (2006) Polymorphism of the protease and reverse transcriptase and drug resistance mutation patterns of HIV-1 subtype B prevailing in China. J Acquir Immune Defic Syndr 42: 512–514
5. Johnson VA, Brun-Vezinet F, Clotet B, et al. (2006) Update of the drug resistance mutations in HIV-1: fall 2006. Top HIV Med 14: 125–130
6. Kantor R, Katzenstein D (2004) Drug resistance in non-subtype B HIV-1. J Clin Virol 29: 152–159
7. Kantor R, Katzenstein DA, Efron B, et al. (2005) Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: results of a global collaboration. PLoS Med 2(4): e112
8. Pereira CF, Paridaen JT (2004) Anti-HIV drug development – an overview. Curr Pharm Des 10: 4005–4037
9. Pieniazek D, Rayfield M, Hu DJ, et al. (2000) Protease sequences from HIV-1 group M subtypes A-H reveal distinct amino acid mutation patterns associated with protease resistance in protease inhibitor-naive individuals worldwide. AIDS 14: 1489–1495
10. Rhee SY, Kantor R, Katzenstein DA, et al. (2006) HIV-1 pol mutation frequency by subtype and treatment experience: extension of the HIVseq program to seven non-B subtypes. AIDS 20: 643–651
11. Shafer RW (2006) Rationale and uses of a public HIV drug-resistance database. J Infect Dis 194: S51–S58
12. Yam WC, Chen JHK, Wong KH, et al. (2006) Clinical utility of genotyping resistance test on determining the mutation patterns in HIV-1 CRF01 AE and subtype B patients receiving antiretroviral therapy in Hong Kong. J Clin Virol 5: 454–457