A low cost duplex polymerase chain reaction to detect common HIV-1 CRF01AE reverse transcriptase inhibitors resistance-associated mutations: 103N/181C and 151M/215Y

Chutitorn Ketloya,b, Sunee Sirivichayakula,c, Kiat Ruxrungthama,c

aVaccine and Cellular Immunology (VCI) Laboratory, Chula Medical Research Center (ChulaMRC), bDepartment of Medicine, Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Background: Two nucleoside plus one non-nucleoside-reverse transcriptase inhibitors (2 NRTIs+NNRTI) combination is prescribed worldwide as a first-line antiretroviral therapy. The cost-effective antiretroviral resistance testing can improve the clinical management of HIV-1 infection.

Objective: To develop a prototype low cost and simple genotypic test to detect common NNRTI- and NRTI-associated RAMs in HIV-1 CRF01_AE in HIV-infected patients.

Methods: The duplex 103N/181C and 151M/215Y/F amplification refractory mutation system (ARMS) were developed and tested with plasma samples from antiretroviral therapy (ART)-naive (group I, n=20) and ART-experienced (>6 months) (group II, n=25) patients whose HIV-1 RNA >1,000 copies/ml in comparison to the standard HIV-1 RT sequencing analysis (ABI, USA).

Results: An excellent concordance (94.3%) was found with the detection limit of duplex ARMS at HIV-1 RNA >1,000 copies/mL. No mutations were observed in the antiretroviral therapy (ART)-naive group (0/20), while 23 out of the 25 (92%) ART-experienced (>6 months) harbored drug-resistant mutant viruses (p <0.01). T215Y/F mutation was the most common genotypic resistance (80%). The NNRTI associated mutations (K103N, Y181C) was found in 6/9 (67%) of patients taking NNRTIs. The NRTI-multidrug resistance (MDR) Q151M was found in 4%.

Conclusion: With the high sensitivity (96%) and specificity (98%), as well as lower cost, this duplex ARMS was valid for further cost-effective HIV drug resistance surveillance study in resource-limited setting. However, clinical uses need further validation in a larger scale study.

Keywords: Amplification refractory mutation system (ARMS), duplex PCR, genotypic resistance, multi-drug resistance, NNRTIs, CRF01_AE, thymidine analog-associated mutations (TAMs).

Resistance to antiretroviral drugs is an important problem for human immunodeficiency virus type 1 (HIV-1) therapy [1]. Antiretroviral resistance testing can improve the clinical management of HIV-1 infection [2, 3]. To date, several phenotypic and genotypic assays for drug resistance have been widely used in developed countries. Phenotypic assays directly measure the degree of viral resistance but they are cumbersome, slow (weeks), and expensive. In contrasts, genotypic assays give faster results [1].

Genotypic resistance assay by HIV genomic sequencing of the polymerase region (Pol) is currently the reference procedure that provides information on all nucleotides on the regions being sequenced. However, this assay is expensive and thus unsuitable for resource-limited setting. The cost of commercially available tests is at least seven-folds higher than that of the monthly expense for antiretroviral therapy (ART) in Thailand. Furthermore, the cost is as high as a whole year cost in most African countries of
ART. Thus, there is a desperate need of a low cost and simple resistance testing for resource-limited settings. Ideally, a low cost, simple point-of-care (POC) drug resistance test is the ultimate goal to improve the earlier detection of treatment failure worldwide.

Simpler procedures to detect the resistance-associated mutation (RAM) codons of interest should be developed for selective polymerase chain reaction (PCR). In this study, the Amplification Refractory Mutation System (ARMS) technique that routinely applied for analysis of genetic mutations [4] has been modified for the development of HIV drug resistance assay. This procedure makes primer more specific by introducing a series of additional mismatches at various sites into the primers that recognized either wild-type (WT) or mutant-type (MT) sequence at the codon of interest [4, 5].

Zidovudine (AZT) resistance mutations (M41L, D67N, K70R, L210W, T215Y/F, and K219E/Q) appear sequentially under treatment pressure [6]. These mutations have been termed “thymidine analog mutations (TAMs)”, based on evidence that beside zidovudine (AZT), stavudine (d4T) also selects them in vivo [7-10]. TAMs are commonly detected among patients who failed the combined two NRTIs (AZT or d4T containing, in particular) [11] and one NNRTI [12]. Furthermore, four TAMs or more mutations showed as cross-resistant within the NRTI class except lamivudine [10]. The first step towards significant resistance is the appearance of T215Y/F mutation. For this reason, most investigators consider this mutation as a marker of AZT resistance for a selective polymerase chain reaction or codon-specific probe hybridization approach.

A set of multi-nucleoside resistance (MNR) (A62V, V75I, F77L, F116Y, and Q151M) [13, 14] was reported to have emerged under combination therapy using nucleoside analogues. These mutations, known as the Q151M complex, confer a much broader resistance pattern, referred to as multiple nucleoside analogue resistance. Because the Q151M substitution appears first, and is thought to be crucial for subsequent development of multiple nucleoside analogue resistance [13], the Q151M mutation has been considered as one of the markers of mutation for the multi-nucleoside resistance. However, the frequency of this mutation is low (<5%) [15, 16].

For non-nucleoside reverse transcriptase inhibitors (NNRTIs), high-level resistance to individual compound can be developed rapidly within a few weeks of initiating monotherapy [17]. The resistance frequently involved only single-point mutation and in many cases, leads to considerable cross-resistance to other NNRTIs. The most common mutations are K103N and Y181C [18, 19]. NNRTI-resistance associated mutations (RAMs) is commonly found among patients who failed NNRTI-based regimen (>55%) [20, 21].

Duplex ARMS for the detection of 103/181 and 151/215 resistance-associated codons makes it possible to test large numbers of samples in a HIV resistance surveillance study because the simplicity, rapidity, and cost-effectiveness of the technique. Therefore, we had developed these assays based on HIV-1 subtype CRF01_AE sequence (Accession No. U54771) and used these assays to determine genotypic resistance of codon 103, 151, 181, and 215 among antiretroviral therapy (ART)-naive and ART-experienced >6 months HIV-1 infected Thais who have plasma HIV RNA >1,000 copies/mL in comparing to the HIV-1 RT sequencing analysis (ABI, USA).

Materials and methods

Control samples

The following control samples were used to evaluate the performances of the HIV RAM-specific ARMS techniques: 1) HIV-1 IIIb viruses were used as the wild type (WT) controls for the amino acid at codon 151(Q), 215(T), 103(K), and 181(Y); 2) WT and 151(M) plasmids were kindly provided by Dr. Hiroaki Mitsuya (NIH, Bethesda, USA); 3) The HIV-1 RT sequencing analysis proven isolates for the mutant (MT) amino acid at codon 103(N), 181(C), and 215(Y/F). Serial dilution of WT HIV-1 IIIb viral stocks (from 500 to 500,000 HIV-1 RNA copies/mL (the standard samples were quantified for HIV-1 RNA by Quantiplex™ HIV RNA assay, version 3.0 (bDNA; Bayer Diagnostics, Emeryville, USA) and WT DNA plasmid (from 500 to 10⁶ copies) were used to evaluate the sensitivity of this assay.

Patient population

Patients who had plasma HIV-1 RNA >1,000 copies/mL from the Immune Clinic of the King Chulalongkorn Memorial Hospital and the Anonymous Clinic, Thai Red Cross AIDS Research Centre were included in this study. Written informed consent approved by the Human Ethics Committee of the
Faculty of Medicine, Chulalongkorn University was obtained from all study patients. The patients were categorized into two groups: Group I: Antiretroviral-naïve patients who had no prior treatment with antiretroviral drugs (ART-naive, n=20), and Group II: Antiretroviral-experienced patients who had received at least two nucleoside analogues and/or non-nucleoside analogues for longer than six months (ART-experienced, n=25)

**RNA separation and RT gene amplification**

HIV RNA was extracted from ethylenediamine tetra-acetic acid (EDTA) plasma by guanidinium isothiocyanate and isopropanol precipitation technique. HIV RT sequence was reverse transcribed with OL primer (5’–GTT CCT TCT GAT GCT TTT TGT CTG–3’, position 2779-2796). The first round of PCR, the conserve region of RT gene (1969-2796) was amplified by OU primer (5’– GTT CCT TCT GA T GCT TTT TGT CTG –3’, position 1969-1990) and OL.

**Duplex amplification refractory mutation system for the detection of HIV-1 RT 103N/181C and 151M/215Y/F resistance-associated codons**

Two duplex ARMS assays for the detection of the K103N/Y181C mutations and Q151M/T215Y/F mutations were developed based on ARMS-based design and the published sequence HIV-1 genotype A/E strain CM240 (Accession No. U54771). The PCR primers were designed by introducing an additional mismatch near the 3’ end. All primers were synthesized by GENSET Singapore Biotech Pte (Singapore).

Details on the two duplex ARMS assays are given in Table 1. Briefly, 3 mL of first-PCR product were used in each of the duplex ARMS assays. The PCR products of 141, 281, 374, and 468 bp were obtained from amplification codon 103, 181, 151, and 215, respectively. 10 mL of the amplification product were visualized on agarose gel electrophoresis after ethidium bromide staining. The genotype of each codon was read directly from the gel by measuring the product band sizes.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primers</th>
<th>PCR conditions</th>
<th>Cycling Conditions</th>
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<tr>
<td>1. 103N/181C ARMS</td>
<td>IU, 103WT&lt;sup&gt;a&lt;/sup&gt; and 181WT&lt;sup&gt;c&lt;/sup&gt; or IU, 103MT&lt;sup&gt;b&lt;/sup&gt; and 181MT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1X-PCR buffer, 1.25 mM MgCl&lt;sub&gt;2&lt;/sub&gt;, 0.4 mM each dNTPs, 10 pmol primer IU, 15 pmol primer 103WT or MT, 5 pmol primer 181 WT or MT, 1.25 Units Taq polymerase</td>
<td>Gene Amp PCR system 9700 pre-denature at 94°C for 5 min. 30 cycles of 94°C for 30 sec, 50.5°C for 30 sec, final extension at 72°C for 7 min.</td>
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<tr>
<td>2. 151M/215Y/F ARMS</td>
<td>IU, 151WT&lt;sup&gt;e&lt;/sup&gt; and 215WT&lt;sup&gt;g&lt;/sup&gt; or IU, 151MT&lt;sup&gt;f&lt;/sup&gt; and 215MT&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1X-PCR buffer, 1 mM MgCl&lt;sub&gt;2&lt;/sub&gt;, 0.4 mM each dNTPs, 10 pmol primer IU, 12 pmol primer 151WT or MT, 1.25 Units Taq polymerase</td>
<td>Gene Amp PCR system 9700 pre-denature at 94°C for 5 min. 30 cycles of 94°C for 30 sec, 72°C for 30 sec, final extension at 72°C for 7 min.</td>
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<sup>a</sup> for amplifying fragment carrying sequence AAA (amino acid K) at codon 103 (5’-TCC CCC ACA TCT AGT ACT GAT GTT-3’).<br><sup>b</sup> for amplifying fragment carrying sequence AAC (amino acid N) at codon 103 (5’-TCC CCC ACA TCT AGT ACT GAT GTT-3’).<br><sup>c</sup> for amplifying fragment carrying sequence TAT (amino acid Y) at codon 181 (5’-ATT GCC GGT GAT CCT TTC ATC ATC-3’).<br><sup>d</sup> for amplifying fragment carrying sequence TGT (amino acid C) at codon 181 (5’-ATCC TAC ATG CGT GTC ATC TTG-3’).<br><sup>e</sup> for amplifying fragment carrying sequence CAG (amino acid Q) at codon 151 (5’-AT ATG GCC GGT GAT CCT TTC ATC ATC TTG-3’).<br><sup>f</sup> for amplifying fragment carrying sequence ATG (amino acid M) at codon 151 (5’-AT ATG GCC GGT GAT CCT TTC ATC ATC TTG-3’).<br><sup>g</sup> for amplifying fragment carrying sequence ACT (amino acid T) at codon 215 (5’-ATG ATG CGT GTC ATC TTG-3’).<br><sup>h</sup> for amplifying fragment carrying sequence TTT (amino acid F) and TAT (amino acid Y) at codon 215 (5’-ATG ATG CGT GTC ATC TTG-3’). Base pairs comprising the codons of interest are in boldface type. The sites of additional mismatches are in shading.
HIV-1 RT Sequencing analysis

Sequencing analysis of RT genes (ABI, USA) as described previously [11] was applied for standard detection of drug resistance mutations in 10 out of the 20 ART-naive patients and all of the ART-experienced patients (n=25) for evaluating the duplex ARMS results. Thus, 35 plasma samples were analyzed.

Statistical analysis

For each codon of interest, results were scored as wild-type and mutant or absence of the interpretable results. Concordance, sensitivity, and specificity of the duplex ARMS were defined by comparison to the standard HIV-1 RT sequencing results. Chi-square test was used to compare genotypic resistance between the ARV-naive and ARV-experienced patients.

Results

Specificity and sensitivity of the 103N/181C and 151M/215Y/F ARMS technique

In an attempt to make duplex ARMS more specific, an additional mismatch was introduced into both WT and MT specific primers (Table 1). The specificity of the wild type (WT) specific primers were also tested with 151(M) plasmid and with known mutant isolates that were observed to be by HIV-1 RT sequencing analysis for each codon. Moreover, the specificity of the mutant (MT) specific primers was tested with WT HIV-1 [IIIb] virus stocks and WT DNA plasmid. The WT and MT specific primers showed the efficient amplification of the target template.

The sensitivity of both duplex ARMS was evaluated with serial dilutions of both WT HIV-1 [IIIb] virus stocks and WT DNA plasmid. Both duplex ARMS had the same detection limit with the RNA (virus stock) (Fig. 1) and DNA (plasmid) template at 1,000 copies/mL.

![Fig. 1](image-url) Determination of sensitivity and specificity of 151M/215Y/F ARMS with serial dilutions of WT HIV-1 [IIIb] virus stocks (measured by Quantrile™ HIV RNA assay, version 3.0, bDNA Bayer Diagnostics, Emeryville, USA). (WT=wild-type, MT=mutant).
Genotypic resistance in HIV-1 infected patients by 103N/181C and 151M/215Y/F ARMS

The 103N/181C (Fig. 2) and 151M/215Y/F (Fig. 3) duplex ARMS were used to evaluate genotypic resistance in all patients. HIV-1 RT sequencing analysis was performed in all of the ART-experienced patients and in 10 randomly selected ART-naive patients. The results of duplex ARMS assays were compared to those generated by HIV-1 RT sequencing analysis.

Comparison of genotypic resistance in HIV-1 infected patients between the 103N/181C and 151M/215Y/F ARMS and the standard HIV-1 RT sequencing analysis

The duplex ARMS were approximately 94% (33/35) concordant with those of the HIV-1 RT sequencing analysis. A significant higher proportion of genotypic resistance was found in the ART-experienced (>6 months) HIV-1 infected patients (group II) compared to the ART-naive patients (group I): 0% (0/20) vs. 92% (23/25) (p<0.01, Chi-square test).

Fig. 2 Examples of the results of 151M/215F/Y ARMS (M=Marker, WT= wild type, MT=mutant) (results from patients No.3 (group I), No.7 (group II), No.8 (group II), and No.12 (group II)).
Among the HIV-1 RT RAMs detected, T215Y/F mutation is the most common genotypic resistance found i.e., 19/25 (76%) by duplex ARMS whereas 20/25 (80%) by HIV-1 RT sequencing analysis. The second most common mutation found was NNRTI associated mutations (overall = 6/25 (24%)): K103N = 4/25 (16%) by duplex ARMS whereas 3/25 (12%) by HIV-1 RT sequencing analysis, and Y181C = 3/25 (12%). However, among the NNRTI failure 67% (6/9) of the patients showed these NNRTI-resistant mutants. The MDR Q151M complex was found only in one patient (4%). Four patients carried viruses with two resistance mutations (Fig. 4).

In ART-experienced patients who had been treated with two NRTIs showed 93% NRTI mutations (one of them is undetectable for 215Y mutation). Mutations at codon 215 were found in 14 (87%) patients (HIV-1 RT sequencing analysis showed 15 (93%)), while codon 151 was detected in one (6%) patient who had AZT/ddI failure. Patients who have been treated with two NRTIs plus NNRTI (triple combination) showed 89% mutations (only one of them showed no mutations). The frequency of the mutations found was 55% (5/9), 44% (4/9), and 33% (3/9) for codon 215, 103, and 181, respectively (Fig. 5).
Fig. 4 Comparison of the detection of RT mutation codons by using the 103N/181C and 151M/215Y/F ARMS (a), and the standard HIV-1 RT sequencing analysis (b) in ART-naïve and ART-experienced of more than six months patients (*Discordant results).
Out of the two discordant results, one was 103N mutation detected by duplex ARMS-PCR but it was 103K (WT) by sequencing, and the other was 215Y mutation detected by the sequencing but was unamplifiable by duplex ARMS.

Comparison to HIV-RT sequencing analysis, the duplex ARMS can detect codon 151 and 181 with the sensitivity and specificity of 100%, whereas it was able to detect codon 103 and 215 a 100 and 95% (19/20) sensitivity and 95% (21/22) and 100% specificity, respectively. The overall sensitivity and specificity of this duplex ARMS-PCR test is 96% and 98% specificity, respectively.

Discussion

Drug resistance is a major cause of antiretroviral failure. Several drug resistance testing have been developed and recommended as a helpful monitoring assay for the management of HIV infection [2, 3]. However, they are still expensive and not widely accessible for developing countries in particular. A rapid, cheap, and less laborious assay is desperately needed. The prototype K103N/Y181C and Q151M/T215Y/F duplex ARMS have been successfully developed in this study.

In Thailand, most individuals are infected with HIV-1 subtype A/E [11, 22]. Much have been reported for genotypic resistance of HIV-1 in North America and Europe where HIV-1 subtype B predominates. Only a few reports related to subtype A/E. The new ARMS primers for all four codons were generated based on HIV-1 CRF01_AE. For the codon 151 and 181, an additional mismatch has been introduced into both WT and MT specific primers. Both primer sets showed the efficient specificity. On the other hand, we have the obstacles for developing the primer set for codon 103 and 215. In an attempt to make codon 103 primer sets more specific, the primes was modified from a mutagenically-separated PCR primer as described previously [23]. Regarding to codon 215, the nucleotide specific sequence between HIV-1 subtype B and CRF01_AE (Thailand) was different. In subtype B, most of the mutations at Thr 215 to Phe or Tyr are changed from ACC to TTC or TAC while most of mutations in CRF01_AE are changed from ACT to TTT to TAT. This observation explains why mutant specific primer by Larder et al. [5] could identify both mutation changes in subtype B, but fail to detect CRF01_AE 215Y mutation in Thai patients (data not shown). This study indicates the limitation of primer design for use in different subtypes. Therefore, the key considerations to develop a successful design of a duplex ARMS depended on several important factors such as the strength and position of nucleotide mismatches in the primer [23, 24], the subtype specific sequences [5], and the resistance-associated mutations.
By using duplex ARMS assay for genotypic resistance evaluation in HIV-1 infected Thais, 94.3% (33/35) showed concordant results with HIV-1 RT sequencing analysis, which is the gold standard. There was significant difference of genotypic resistance between ART-naive (group I) and ART-experienced of more than six months HIV-1 infected patients (group II) (p < 0.01, Chi-square test). There is no mutation in the ART-naive patients, while 23 out of the 25 (92%) ART-experienced patients harbored drug-resistant mutant viruses. The most common mutation occurred at codon 215 (76%) since most of the ART-experienced patients were treated with AZT in combination therapy and no mutation were found at codon 103 or 181 in patients who have been treated with NRTIs only. These findings confirm that HIV resistance occurs under selective pressure of antiretroviral drugs and emphasizes that resistance testing is helpful to guide switching regimens in patients who fail therapy [3, 25]. Of note, Q151M mutant is uncommon (1/25 or 4% in NRTI failure) and found only in ddI failure. This finding is similar to our previous report [11].

Out of the two discordant results, one was 103N mutation detected by duplex ARMS-PCR but it was 103K (WT) by sequencing, and the other was 215Y mutation detected by the sequencing but was unamplifiable by duplex ARMS. This may due to a greater sensitivity of duplex ARMS. Since this patient was treated with EFV in his combination drugs, the K103N could be occurred. To prove this explanation, a more sensitive assay to detect the minority of RAMs such as the allele-specific real-time PCR [26] is required. However, it is also possible that it could be a result of mispriming or false positive. The second discordance was 215Y mutation by sequencing but was unamplifiable by duplex ARMS. It might be a result of inefficiency of the 215 mutant specific primers. Thus, the issues of minority of mutants in the samples, amplifying errors from a tiny volume of blood for both duplex ARMS and sequencing may explain such discordance.

In terms of sensitivity of the detection, both the duplex ARMS-PCR and the standard sequencing assays have the same detection limit at 1,000 HIV-1 RNA copies/mL. The main limitation of duplex ARMS is that it could only give information of only these four codons (103, 151, 181, and 215), whereas HIV-1 RT sequencing analysis could provide the information of the entire HIV-1 RT region sequenced. However, sequencing analysis is too costly for wild-spread use in developing countries such as Thailand. In contrast, the cost of the duplex ARMS is less than 11 $US per sample and the time consuming is only eight hours (see Table 2).

As these duplex ARMS are the prove-of-concept prototypes, the major limitation is only four codons (103, 151, 181, and 215) can be detected by this assay. Ideally, it should cover the other most common mutations found in HIV-infected Thais, which generally used GPO-VIR based regimen (a combination of stavudine, lamivudine, and nevirapine). For example, M184V for 3TC (89%), other TAMs such as D67N and K70R (24% and 17%), other NNRTIs such as G190A and V108I (24% and 14%) [27]. Further development for a point-of-care HIV-1 drug resistance genotype is warranted to be investigated to serve resource-limited settings when patients failed GPO-VIR based regimen. Nevertheless, it is very unlikely that such an approach will suit a setting when patients have been experienced to more than three classes of antiretroviral therapy.

### Table 2. Comparison of 103N/181C and 151M/215Y/F ARMS with HIV-1 RT sequencing analysis.

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<th>103N/181C and 151M/215Y/F duplex ARMS</th>
<th>HIV-1 RT sequencing analysis</th>
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<tr>
<td>Minimal viral load cut-off</td>
<td>1,000 copies/mL</td>
<td>1,000 copies/mL</td>
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<tr>
<td>Methodology</td>
<td>Comparable assay</td>
<td>Gold standard assay</td>
</tr>
<tr>
<td>Number of mutation detection</td>
<td>only four codons</td>
<td>all</td>
</tr>
<tr>
<td>Cost</td>
<td>$US 11</td>
<td>$US 55</td>
</tr>
<tr>
<td>Time consuming of the assay</td>
<td>eight hours</td>
<td>two days</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>96%</td>
<td>Gold standard</td>
</tr>
<tr>
<td>Specificity</td>
<td>98%</td>
<td>Gold standard</td>
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This does not include the fixed cost of labor and instrument maintenance.
Conclusion

Although genotypic resistance assay by HIV-1 RT sequencing analysis is currently the reference procedure to provide information on all nucleotides on the regions being sequenced, this assay is expensive and thus unsuitable for resource-limited countries. The 103N/181C and 151M/215Y/F ARMS were able to detect NNRTIs and multi-NRT resistant mutants comparable to the standard HIV-1 RT sequencing analysis. With the high sensitivity (96%) and specificity (98%), as well as lower cost, these duplex ARMS assays are thus valid for further cost-effective HIV drug resistance surveillance study in resource-limited countries. However, clinical uses in detection of NNRTIs and multi-NRT resistances need further investigation in a larger scale study. In addition, this duplex ARMS can be a prototype for further development of point-of-care HIV-1 drug resistance testing for GPO-VIR based failure in the future.

Acknowledgements

This work was supported by a grant from the AIDS Division Bureau of AIDS, TB and STDs, Department of Disease Control, Ministry of Public Health, Thailand, and by the Research Team Strengthening Grant from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The authors declare no conflict of interest.

Abbreviations

ARMS = Amplification refractory mutation system,
AZT = zidovudine,
MDR = multi-drug resistance,
NAMs = nucleoside analogue mutations,
NNRTIs = non-nucleoside reverse transcriptase inhibitors,
NRTIs = nucleoside reverse transcriptase inhibitors,
RAMs = resistance-associated mutations.

References


