Asian Biomedicine Vol. 5 No. 2 April 2011; 249 - 255 DOI: 10.5372/1905-7415.0502.032

Original article

Comparison of in-house HIV-1 genotypic drug resistant test with commercial HIV-1 genotypic test kit

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Background: The use of combination antiretroviral therapy (cART) has become a standard of care in the treatment of HIV infection. However, antiretroviral drug resistance occurs in a substantial number of patients. In resource-limited settings, genotypic resistance assay using a commercial kit is costly.

Objective: Focus on the validation of an in-house HIV-1 specific genotypic drug resistance assay in Thai patients failing cART.

Materials and methods: Results of HIV-1 genotypic drug resistance assay was evaluated by comparing an in-house method to a commercial test. The TRUGENE *HIV-1* genotyping kit was used in 79 plasma specimens (49 from HIV patients failing cART therapy and 30 from proficiency testing panels).

Results: The results from the in-house assay were comparable to those obtained from the TRUGENE *HIV-1* genotyping kit with >99.0% codon-to-codon agreement. The lower limit of detection by the in-house assay was approximately 100 copies/mL of HIV-1 RNA. In addition, this in-house assay would allow testing of samples from patients infected with HIV-1 subtype other than B.

Conclusion: The in-house HIV-1 genotypic drug resistance assay may be used as an alternative to commercial kits, particularly in resource limited settings.

Keywords: Antiviral therapy, drug resistance, genotype, HIV-1, in-house assay

The use of combination antiretroviral therapy (cART) has become a standard of care in the treatment of HIV infection. In Thailand, cART is free of charge to all HIV-infected patients who have the indication for treatment since 2003 [1, 2]. After widespread use of cART, treatment failure from the development of antiretroviral (ARV) drug resistance has occurred in many patients [3, 4]. Selection of an effective second or third-line regimen in patients who have failed the initial cART regimen is usually difficult and requires HIV-1 drug resistance testing [5, 6].

Internationally, evaluation and monitoring of ARV drug resistance is recommended in all patients with HIV infection [7-9]. The World Health Organization (WHO) recommends that resistance testing should be performed for drug resistance surveillance purposes rather than individual patient monitoring in resource-limited setting [10] due to the high cost of the test.

Most genotyping methods detect HIV drug resistance by sequencing the protease (PR) and reverse transcriptase (RT) regions of the *pol* gene. Currently, two commercial genotyping resistance systems approved by the United States Food and Drug Administration (FDA) are the ViroSeq™ HIV-1 Genotyping System (Applied Biosystems, Foster City, USA) and TRUGENE *HIV-1* genotyping kit (Visible Genetics Inc, Toronto, Canada). In-house genotypic HIV-1 drug resistance tests with low running costs have been designed [11-15]. Most of them have high concordance with commercial genotyping systems. Commercial and "in-house" sequencing methods are characterized by manual viral RNA extraction steps [16, 17] and often have a nested amplification step

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[11, 12]. Recent studies showed that commercial HIV-1 genotyping systems are applicable to various HIV-1 subtypes, but some problems with non-B subtypes have been reported [16, 18-21].

Analysis of drug resistance in non-B subtypes of HIV-1 is becoming more important because the availability and use of ARV drugs are growing throughout the world [22]. The circulating recombinant form AE (CRF01_AE) is the most prevalent strain circulating in Thailand and other Southeast Asian countries [23-25].

In this study, we evaluated the possibility of using an in-house HIV-1 genotypic drug resistance testing system for the monitoring of ARV drug resistance in patients with HIV infection in Thailand. Focus was put on the validation of an in-house HIV-1 specific genotypic drug resistance assay in Thai patients failing cART. The results of the in-house assay were compared with those of the TRUGENE *HIV-1* genotyping kit.

Materials and methods

This study was approved by the Human Ethics Committee of the Faculty of Medicine, Chiang Mai University.

Clinical specimens

Seventy-nine plasma specimens (62 CRF01_AE and 17 subtype B) including specimens from patients failing their cART (n = 49)) and those from proficiency testing panels (n = 30) were used to compare an in-house genotypic drug resistance assay with the TRUGENE *HIV-1* genotyping kit. The HIV-1 RNA level (viral load) of these specimens ranged from >1,000 to 600,000 copies/mL. Specimens from proficiency testing panels consisted of those from an external quality assurance (EQA) panels of the Thailand National Institutes of Health (NIH) (n = 15) and from the Treat Asia Quality Assessment Scheme (TAQAS) of the Australian National Serology Reference Laboratory (NRL) (n = 15). Nineteen additional plasma specimens from patients on cART with low plasma HIV-1 RNA levels (64 to 865 copies/mL) were used to determine the lower limit of detection of the in-house assay. Ten other specimens from naïve patients with known HIV-1 subtypes (7 CRF_01/B, 1 CRF_01/C, and 2 subtype C) were tested by the in-house assay to evaluate its capability to amplify and sequence HIV-1 intersubtype recombinant forms as well as other subtypes found in Thailand. All plasma HIV-1 RNA levels were determined using the AMPLICOR HIV-1 MONITOR test, version 1.5 (Roche Diagnostics, Branchburg, USA). All laboratory tests were performed at the Faculty of Medicine and Research Institute for Health Sciences, Chiang Mai University except for the TRUGENE *HIV-1* genotyping tests, which were done at a commercial laboratory.

RNA extraction and polymerase chain reaction (PCR)

The *pol*-PR and *pol*-RT gene were used as the templates in amplification reaction. The specific sequence primers used in this in-house assay were derived from a method that was previously described [26-28]. The primers covered the whole protease gene (PR, 1-99 amino-acid base sequence) and part of the reverse transcriptase gene (RT, 1-260 amino-acid base sequence). In each extraction and PCR, a pool of HIV positive plasma samples was included as a positive control. Normal human plasma specimens were used as a negative control. The viral RNA was extracted from 150 µL of individual plasma sample using a NucleoSpin® Viral Isolation kit (Macherey-Nagel, Duren, Germany). The single stranded HIV RNA was reverse transcribed and then amplified with use of the one step RT-PCR technique using Superscript□ III one step RT-PCR with platinum *Taq* (Invitrogen, Carlsbad, USA). In this step, the outer primer pairs used for the PR gene were DRPRO5 (5′- AGACA GGYTAATTTTTTAGGGA) and DRPRO2L (5′- TATGGATTTTCAGGCCCAATTTTTGA). The outer primer pairs of DRRT1L (5′- ATGATAGG GGGAATTGGAGGTTT) and DRRT4L (5′- TA CTTCTGTTAGTGCTTTGGTTCC) were used for the RT gene. The cycling condition for the one step RT-PCR was 55°C for 20 minutes, 95°C for two minutes [94°C 15s/52°C 15s/72°C 45s] x 40, and 72°C for five minutes. The DNA was then amplified by nested PCR using the KOD DNA polymerase kit (Toyobo, Tokyo, Japan). The inner primers, DRPRO1M (5′- AGAGCCAACAGCCCCACCAG) and DRPRO6 (5′- ACTTTTGGGCCAT CCATTCC), were used for the PR gene and the primers, DRRT6L (5′- TAATCCCTGCATAA ATCTGACTTGC) and DRRT7L (5′- GACCTA CACCTGTCAACATAATTGG) were used for the RT gene for this amplification. A 464 base pairs (bp) fragment of the PR gene and an 888 bp of the RT gene were amplified. The cycling condition for the

nested PCR step was 94°C for 15 seconds [94°C 5s/ 60°C 3s/74°C 30s] x 30, and 72°C for seven minutes.

DNA sequencing

The nested PCR products were purified with NucleoSpin® extract II (Macherey-Nagel, D□ren, Germany) and sequenced in both directions with 1/10 dilution of the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) containing four specific primers. Two primers, DRPRO1M and DRPRO6, were used to analyze the nucleotide sequence on the PR gene. Two other primers, DRRT6L and DRRT7L, were used to analyze the RT gene. These primers provided overlapping and bidirectional sequences covering the region where all defined protease and reverse transcriptase inhibitor resistance-related mutations were positioned. The pGEM control (Applied Biosystems, Foster City, USA) was processed along with the samples as a positive sequencing control. Excess dye terminator after cycle sequencing was removed by the EDTA/ethanol precipitation method before loading into the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). The nucleotide sequences were obtained and edited using SeqScape software V2.6 (Applied Biosystems, Foster City, USA). As the software that comes with the TRUGENE *HIV-1* genotyping kit compares the consensus sequence with a reference, HXB2, to determine mutations present in the sample, in-house assay used the same reference sequence strain HXB2 (K03455) during manual sequences editing. All sequences were analyzed for HIV-1 specificity by using BLASTN version 2.2.23 (National Center for Biotechnology Information, USA) and interpreted for mutation point, drug resistance, and subtype using the Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/pages/algs/HIVdb.html) and International AIDS Society-USA mutation panel [29].

Sequencing primers for intersubtype recombinant forms

The in-house HIV-1 genotypic drug resistance assay was evaluated for its capability to amplify and sequence HIV-1 intersubtype recombinant forms and other subtypes found in Thailand. Ten clinical specimens with a known HIV-1 subtype from na□ve patients were tested (7 CRF_01/B, 1 CRF_01/C, and 2 subtype C).

Lower limit of detection of the in-house assay

The lower limit of HIV-1 RNA detection was evaluated by testing 19 plasma specimens derived from treated patients with HIV-1 RNA range from 64 to 865 copies/mL.

Statistical analysis

Results from the TRUGENE *HIV-1* genotyping assay were used to evaluate the performance of our in-house assay as the gold standard. The percentage codon agreement and 95% confidential interval (CI) were calculated using the number of mutations genotyped by the in-house technique, divided by the number of mutations genotyped by the commercial assay. The number of mutations genotyped by the in-house method, but not by the TRUGENE *HIV-1* genotyping assay was not counted.

Results

General characteristics of 49 clinical samples

Clinical characteristics including age, sex, current CD4+ cell count, current HIV-1 viral load, nucleoside reverse transcriptase inhibitor (NRTI) backbone, and HIV-1 subtype are shown in **Table 1**.

Comparison of in-house genotypic HIV-1 drug resistance testing with the TRUGENE HIV-1 genotyping kit

From the 79 plasma specimens tested, 100% were successfully genotyped by the in-house assay, whereas 77 (97.5%) were successfully genotyped by the TRUGENE *HIV-1* genotyping assay. Among 77 nucleotide sequences genotyped by both assays, 84.4% were completely concordant. The mean percentage of codon agreement between the in-house and TRUGENE *HIV-1* genotyping assay was 99.8% (95% CI: 99.4, 100.2). In the remaining 12 nucleotide sequences with partial codon-to-codon agreement, there were 29 ambiguous mutation points (**Table 2**). This could be explained by the presence of nucleotide mixtures at the designated positions, and interpreted as either wild type or mutant. Sixty codons (36 PR codons and 24 RT codons) on each sequence that were drug resistance-associated mutations were identified. Of the 77 samples that were successfully genotyped by both assays, the overall codon-to-codon agreements were 99.4% and 99.2% for PR codons and RT codons, respectively. Interestingly, in two of

these 12 sequences with ambiguous mutation points, T69insertion, was identified by the in-house assay, but not by the TRUGENE *HIV-1* genotyping assay. Two of 79 (2.5%) specimens successfully amplified by the in-house assay, but not by TRUGENE *HIV-1* genotyping assay, had 1,130 and 6,080 copies/ml of HIV-1 RNA**.** The nucleotide sequence showed that mutations in the RT gene associated with resistance to reverse transcriptase inhibitors (RTIs) were detected in both specimens. The first specimen had mutations at D67N, M184V, K103N and Y181C. The second specimen had D67N, K70E, M184V, K101E, V108I and Y181C. Both specimens were HIV CRF01_AE strains.

Table 1. Characteristics of 49 HIV-infected patients at the time of first cART regimen failure.

Characteristics	Number[a]
Age, mean (range)	38.0 (18-70)
Sex	
Male	21 (42.9%)
Female	27 (55.1%)
Transgender	1 (2.0%)
NRTI[b] backbone	
Stavudine and lamivudine	38 (77.6%)
Zidovudine and lamivudine	7 (14.3%)
Didanosine and lamivudine	1 (2.0%)
Tenofovir and lamivudine	1 (2.0%)
Zidovudine and didanosine	2 (4.1%)
Third drug	
Nevirapine	31 (63.3%)
Efavirenz	12 (24.5%)
PI[c]	6 (12.2%)
Median plasma HIV-1 RNA, copies/ml [IQR][d]	75001 [28000, 100000]
Median CD4+ cell count, cells/mm^3 [IQR]	122 [56, 178]
Median percentage of CD4+ cell, % [IQR]	6.0 [4.0, 9.5]
HIV-1 subtype	
CRF01_AE	47 (95.9%)
B	2 (4.1%)

[a]number (%) of patients, unless otherwise indicated, [b]nucleoside reverse transcriptase inhibitor. [c]protease inhibitor, [d]interquatile range.

Table 2. Partially discordant drug resistant mutations detected by the in-house assay and TRUGENE *HIV-1* genotyping kit (TG).

Sample code	*pol* subtype	Number of mutations detected by in-house		Number of mutations detected by TG	
		PR[a]	RT[b]	PR	RT
S19	CRF01_AE	8 (22.2%)	8 (33.3%)	8 (22.2%)	6 (25.0%)
S25	CRF01_AE	5 (13.9%)	8 (33.3%)	5 (13.9%)	9 (37.5%)
S30	CRF01_AE	8 (22.2%)	5 (20.8%)	6 (16.7%)	4 (16.7%)
S37	CRF01_AE	6 (16.7%)	8 (33.3%)	6 (16.7%)	7 (29.2%)
S41	CRF01_AE	5 (13.9%)	1 (4.2%)	5 (13.9%)	0
S52	CRF01_AE	10 (27.8%)	6 (25.0%)	11 (30.6%)	6 (25.0%)
S53	CRF01_AE	11 (30.6%)	8 (33.3%)	9 (25.0%)	8 (33.3%)
S57	CRF01_AE	10 (27.8%)	7 (29.2%)	7 (19.4%)	6 (25.0%)
S59	CRF01_AE	14 (38.9%)	8 (33.3%)	11 (30.6%)	8 (33.3%)
S63	CRF01_AE	7 (19.4%)	6 (25.0%)	5 (13.9%)	6 (25.0%)
S65	CRF01_AE	8 (22.2%)	7 (29.2%)	7 (19.4%)	5 (20.8%)
S74	B	7 (19.4%)	3 (12.5%)	5 (13.9%)	2 (8.3%)

[a]protease, [b]reverse transcriptase.

Sequencing primers for intersubtype recombinant forms

The in-house assay was used to test plasma samples from 10 na□ve patients infected with known HIV-1 subtypes (7 CRF_01/B, 1 CRF_01/C, and 2 subtype C) to evaluate for its capability to amplify and sequence HIV-1 other subtypes found in Thailand. All 10 samples were successfully amplified and sequenced on both the PR and RT genes by the in-house assay.

Lower limit of detection of the in-house assay

Nineteen plasma samples (18 CRF01_AE and 1 subtype B) with HIV-1 RNA level ranging from 64 to 865 RNA copies/mL were tested. Fifteen samples were successfully amplified and genotyped by the in-house assay. Drug resistance-related mutations in the RT regions were identified in five samples. The remaining 10 specimens were wild type. Four of the 15 samples had HIV-1 RNA <100 copies/mL. All 15 plasma samples (4 HIV-1 RNA <100 copies/mL and 11 HIV-1 RNA >100 copies/mL) were reproducibly amplified for both PR and RT genes by the in-house assay. The four samples that could not be amplified by in-house assay had HIV-1 RNA 64, 65, 74 and 83 copies/mL. Therefore, the detection limit of this in-house assay is approximately 100 copies/ml of HIV-1 RNA.

Discussion

We evaluated the in-house HIV genotypic drug resistance test using 49 plasma samples from patients who had failed their first line cART regimen and 30 specimens from proficiency testing panels. The overall results from the in-house assay were comparable to those obtained from the commercial test kit, TRUGENE *HIV-1* genotyping assay, with >99.0% codon-to-codon agreement. Two specimens that were successfully amplified by in-house assay but not by TRUGENE *HIV-1* genotyping assay, revealed mutations associated with resistance to RTIs leading to a decision by the attending physician to change cART regimen. The results also confirmed finding from previous reports that the commercial HIV-1 genotyping systems had problems with non-B strains [16, 18-21].

In the study of 19 samples with known HIV-1 RNA levels, the lower detection limit of the in-house assay was approximately 100 copies/mL of HIV-1 RNA. The higher sensitivity of DNA amplification by the in-house test is probably due to the use of nested-PCR methodology and/or higher capability of in-house primers to amplify and sequence non-subtype B HIV-1, as observed in other studies [11-13]. A study of 10 plasma specimens with known HIV subtypes also indicated that our in-house drug resistance assay could be used for HIV-1 subtypes other than CRF01_AE and subtype B. Commercial genotyping systems do not recommend the use of their assay for samples with <1,000 copies/mL of HIV-1 RNA [9, 14, 17]. This recommendation is because the success rate of testing samples with HIV-1 RNA <1,000 copies/mL is low and on the assumption that amplification of those samples might lead to the selection of non-representative strains. However, many studies suggested that HIV-1 genotypic resistance testing is still informative for patients with low viral burden [14, 30]. Major resistance mutations were commonly detected in samples with <1,000 copies/mL of HIV-1 RNA [30]. The present result indicated that genotyping performed as soon as treatment failure was suspected. Although the viral burden was still low, the result may be meaningful. In a subset of five patients with HIV-1 RNA between 129 and 865 copies/mL, resistance against one or more components of the cART regimen was detected (data not shown).

One important aspect of our in-house program was the participation of external quality assurance (EQA) schemes, namely the Thai NIH and TAQAS [31] which have contributed to the consistency and standardization of our assay. The in-house assay successfully amplified all the 30 EQA samples. The performance of our laboratory showed high agreement at the level of nucleotide editing and detection of drug resistant mutations. The mutation patterns detected were comparable to those in other certified laboratories that participated in the quality assurance schemes, resulting in our in-house assay being certified by the Thai NIH and TAQAS.

Our in-house drug resistance test is a viable alternative to commercial tests in resource-limited settings. Initial investment is needed to train technicians in molecular biology methods, use of the Stanford HIV database, and other facilitating software such as SeqScape, as well as basic equipment. However, procedure requiring more expensive equipment (DNA sequencing) can be inexpensively outsourced. This makes the total unit costs 50% to one-third of those currently available from using commercial test kits. In addition, one central drug resistance testing facility may

be able to serve the whole country. The high sensitivity of our in-house test with the detection limit of 100 copies/mL HIV-1 RNA level may permit its use as a qualitative screening test for plasma HIV-1 RNA level in patients with suspected cART failure, thus bypassing use of the viral load test.

In conclusion, the in-house HIV-1 genotypic drug resistance assay is highly cost-effective and can be used as an alternative to commercial kits, particularly in resource limited settings.

Acknowledgments

We wish to thank the National Research University Project under Thailand's Office of the Higher Education Commission and the Graduate School, Chiang Mai University for financial support. We gratefully acknowledge Dr. Wataru Sugiura (AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan), Professor Koya Ariyoshi (Global COE Program/Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan), Professor Praphan Phanuphak, Professor Kiat Ruxrungtham and Dr. Sunee Sirivichayakul (Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand), and Dr. Siriphan Saeng-Aroon (Ministry of Public Health, Thailand) for their support of the study.

The authors do not have any conflict of interest.

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