Original Article

Clinical Evaluation of an In-House Human Immunodeficiency Virus (HIV) Genotyping Assay for the Detection of Drug Resistance Mutations in HIV-1 Infected Patients in Singapore

Kuan Kiat Chew, [1]*BSc(Hons)*, Kah Ying Ng, [1]*MSc*, Wei Xin Khong, [1]*PhD*, Palvinder Kaur, [1]*BMedSc*, Joe Kwan Yap, [1]*Dip(Mol Biotechnol)*, Arlene Chua, [1]*MD, MSc*, Mei Ting Tan, [1]*BMedSc*, Yin Ling Koh, [2]*MBBS, MRCP*, Koh Cheng Thoon, [3]*MBBS, MMed, MRCPCH*, Yee Sin Leo, [1]*MBBS, MMed, MRCP*, Oon Tek Ng, [1]*MRCP, FAMS, MPH*

Abstract

Introduction: Human immunodeficiency virus type 1 (HIV-1) genotyping resistance test (GRT) is essential for monitoring HIV-1 drug resistance mutations (DRMs). High cost and HIV-1 genetic variability are challenges to assay availability in Singapore. An in-house Sanger sequencing-based GRT method was developed at the Communicable Disease Centre (CDC), Singapore's HIV national treatment reference centre for both subtype B and non-subtype B HIV-1. **Materials and Methods**: The in-house GRT sequenced the first 99 codons of protease (PR) and 244 codons of reverse transcriptase (RT) in the *pol* gene. The results were compared with the Food and Drug Administration (FDA)-approved ViroSeq™ HIV-1 Genotyping System. **Results**: Subtype assignment for the 46 samples were as follows: 31 (67.4%) CRF01_AE, 14 (30.5%) subtype B and 1 (2.1%) subtype C. All 46 samples had viral load of ≥500 copies/mL, and were successfully amplified by the in-house primer sets. Compared to the ViroSeq™ test, our in-house assay showed drug-resistance conferring codon concordance of 99.9% at PR and 98.9% at RT, and partial concordance of 0.1% at PR and 1.1% at RT. No discordant result was observed. **Conclusion**: The assay successfully identified DRMs in both subtype AE and B, making it suitable for the efficient treatment monitoring in genetically diverse population. At less than half of the running cost compared to the ViroSeq™ assay, the broadly sensitive in-house assay could serve as a useful addition to the currently limited HIV genotyping assay options for resource-limited settings, thereby enhancing the DRM surveillance and monitoring in the region.

Ann Acad Med Singapore 2012;41:553-8

Key words: Drug resistance mutations, Genotypic resistance test, HIV-1

Introduction

Human immunodeficiency virus (HIV) drug resistance testing is a standard of care test recommended for HIV-infected patients prior to initiation of antiretroviral therapy (treatment-naïve), and prior to treatment switch (treatment-experienced).[1-3] For treatment-naïve patients, HIV drug resistance testing detects transmitted resistance and enables selection of an optimal first regimen. For treatment-experienced patients, drug resistance testing facilitates selection of an optimal next regimen with the highest number of effective agents.

Two types of resistance assays, genotypic and phenotypic, are commonly used to determine sensitivity or resistance of HIV isolates to individual antiretroviral drugs (ARVs).[4] In HIV, key mutations that confer drug resistance often arise at positions that encode for the viral protease (PR) and reverse transcriptase (RT).[5] Currently available commercial genotyping resistance systems, such as the ViroSeq™ genotyping system version 2.0 (Celera Diagnostics, CA, USA) and TRUGENE™ HIV-1 genotyping system (Siemens Healthcare Diagnostics, NY, USA) leverage deoxyribonucleic acid (DNA) sequencing technology to detect resistance mutations in the PR and RT region of the *pol* gene.[6,7]

Despite the pivotal role of genotypic resistance testing (GRT) assays to ensure ongoing regimen efficacy, the prohibitive high running cost of these commercial genotyping assays have impeded their routine application in Singapore. Furthermore, HIV-1 genetic diversity is a key challenge to the local adaptation of these commercial assays. Commercial kits were primarily designed for use in affluent industrialised countries, and are optimised for subtype-B viruses.[8-10] In Singapore, the predominant circulating strains are CRF01_AE, and subtype B.[11] Recently, we described

[1]Department of Infectious Disease, Tan Tock Seng Hospital, Singapore
[2]Department of Infectious Disease, Singapore General Hospital, Singapore
[3]Department of Paediatric Medicine, Infectious Disease Service, KK Women's and Children's Hospital, Singapore
Address for Correspondence: Dr Ng Oon Tek, Department of Infectious Disease, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433.
Email: oon_tek_NG@ttsh.com.sg

a further novel circulating recombinant form (CRF), CRF51_01B, which accounted for 12% of infections in a recent prevalence study.[12] A cost-effective GRT assay that is readily adaptable to non-B subtypes is crucial to allow baseline and detection of treatment-failure HIV drug resistance genotyping in Singapore.[2,13]

Recognising the pressing need for an affordable GRT in our region, an inexpensive and sensitive Sanger sequencing-based GRT method was developed at the Communicable Disease Centre (CDC), Singapore's HIV national treatment reference centre. Here, we present data on the accuracy of the in-house HIV GRT assay. Clinical evaluations of the in-house system were performed by comparing our results with those of the Food and Drug Administration (FDA)-approved ViroSeq™ Genotyping System. Additionally, the initial experience with implementing this test for clinical use is described.

Materials and Methods

Clinical Specimens

In the initial pilot of the in-house assay for clinical care, parallel testing using the in-house GRT and the ViroSeq™ genotyping system was performed for quality assurance on 41 patient samples. A further 5 samples from the Treat-Asia Quality Assurance Scheme (TAQAS) HIV-1 Genotypic Drug Resistance External Quality Assessment Scheme were added to this analysis. The National Healthcare Group ethics committee approved this study (Reference number: E/2012/00438).

Ribonucleic acid (RNA) Extraction

Patient plasma were separated from EDTA blood samples and stored at -80°C. Prior to ribonucleic acid (RNA) isolation, viral particles from either 1 mL of patient plasma or 0.5 mL of proficiency testing plasma was concentrated by ultra-centrifugation at 24,000 x g (relative centrigual force (RCF)) for an hour at 4°C. RNA, genotyped by the ViroSeq™ system, was extracted as according to manufacturer's recommendations and re-suspended in 50μL of RNA diluent. In subsequent routine testing of patient specimens and proficiency testing, RNA was isolated by the automated system MagNa Pure Compact using the RNA isolation kit (Roche, Switzerland).

Reverse Transcription and Polymerase Chain Reaction (PCR)_Amplification

For the ViroSeq™ system, RNA samples were reverse-transcribed and complementary DNA (cDNA) amplified as according to manufacturer's recommendations. For the in-house GRT assay, 5 μL remnant RNA was reverse-transcribed and cDNA amplified using the Qiagen One-Step RT-PCR kit (Qiagen, Germany). The sequences of the amplification primers were: forward primer A: 5'- GAA CAG ACC AGA GCC AAC AGC CCC ACC-3' from HXB2 nucleotide positions 2139 to 2165; reverse primer C1.1: 5'-TTT GAC TTG CCC AAT TTA GTT TTC CCA C-3' and C1.2: 5'- TTT GAC TTG CCC AAT TTA ACT TTC CCA C-3' from HXB2 nucleotide positions 3330 to 3357 (Fig. 1). Five microlitre of the extracted RNA was added to a 5 μL reverse transcriptase-polymerase chain reaction (RT-PCR) reaction mix consisting of the following: 2 μL 5x Qiagen One-step RT-PCR buffer, a final concentration of 0.4 mM of dNTPs, a final of 0.2 μM of each forward and reverse primers, and 0.5 μL enzyme mix. Thermal cycling conditions for RT-PCR were as follows: 50°C for 30 minutes, 95°C for 15 minutes, 42 cycles of [94°C 10 s/65°C 60 s/68°C 1 min 45 s] and a final extension at 68 °C for 5 minutes.

Sequencing

PCR products from either the ViroSeq™ system or in-house GRT assay were treated with ExoSAP-IT prior to cycle sequencing. The sequencing primers consisted of primer A, C1.1, C1.2 and the following additional primers: forward primer B1.1: 5'- AAT TAG CCC TAT TGA CAC TGT ACC A-3' and B1.2: 5'- CAT TAG TCC TAT TGA AAC TGT ACC A-3' from HXB2 nucleotide position 2552 to 2576 and reverse primer D: 5'- TTG CCG GTG ATC CTT TCC ATC C-3' (Fig. 1).
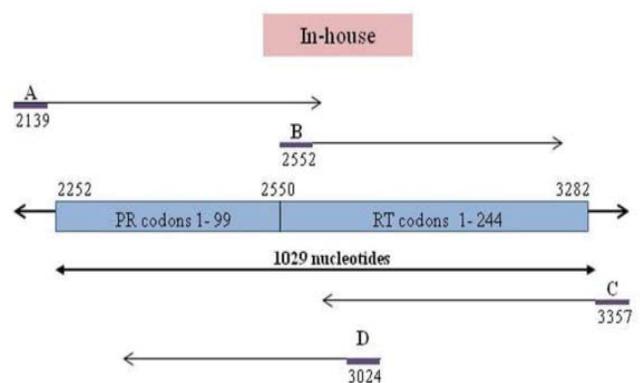

Fig. 1. Schematic diagram of PR and RT regions covered by in-house HIV genotyping assay, showing location of primers and amplification regions.

Cycle sequencing for both ViroSeq™ and in-house GRT assay used universal conditions as described in the ViroSeq™ manufacturer's recommendations. Sequencing by the in-house GRT assay was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit version

3.1 (Life Technologies, USA) with specified primers. All extension products were purified by magnetic beads and sequenced on the 3730xl DNA Analyzer (Life Technologies, USA) at IMCB, Singapore.

Interpretation and Data Analysis

For the ViroSeq™ system, individual sequence fragments were aligned and edited as with the ViroSeq™ software package version 2.8. For the in-house GRT assay, sequence fragments were aligned and edited with the Staden Package.[14] Nucleotide sequence editing was performed as per the guidelines of ViroSeq™. All consensus pol sequences were exported and submitted to Stanford University HIV Drug Resistance HIVdb program version 6.2.0 (http://sierra2. standford.edu/sierra/servlet/Jsierra) for genotypic resistance interpretation. HIV-1 genotype assignment was based on the Stanford report.

Identification of HIV drug resistance positions in each sample was based on an analysis of 50 codons (17 PR and 33 RT), as listed in the 2011 International AIDS Society consensus statement on drug-resistance mutations (DRMs).[5] A total number of 2300 codons [782 PR (17 x 46) and 1518 RT (33 x 46)] were analysed and grouped under the following categories: (i) Concordant, when both assays identified the same amino acid; (ii) Partially concordant, when amino acid mixture was detected by 1 assay but not by other and (iii) Discordant, when 2 assays detected different amino acids.[15]

Phylogenetic Analysis

Pol gene sequences of each patient sample from both assays, including samples from proficiency testing, were imported into Maximum Composite Likelihood Method (MEGA), version 5.[16] All positions containing gaps were manually removed and aligned using the software. Phylogenetic tree was constructed using Neighbour-Joining method, with bootstrap analysis of 500 replicates. The evolutionary distances were computed using the MEGA.[17]

Correlation Between Drug Resistance Mutations (DRM) Patterns and Treatment Regimens

To evaluate the correlation between the drug resistance mutations (DRM) patterns and treatment regimens for 33 subjects with available treatment history record, ARVs used in the latest and previous treatments were first classified into 3 main classes: nucleoside RT inhibitor (NRTI), non-nucleoside RT inhibitor (NNRTI) and protease inhibitor (PI). This was followed by examining the expected DRM patterns in response to a specific class of ARV.

Results

Subtype assignment for the 46 samples were as follows: 31 (67.4%) CRF01_AE, 14 (30.5%) subtype B and 1 (2.1%) subtype C. All 46 selected clinical specimens had viral loads of ≥500 copies/mL, and were successfully amplified and sequenced by both ViroSeqTM and in-house assays. A total number of 2300 DRMs and 13,478 non-DRMs codons in both PR and RT regions were analysed.

Of the 2300 DRM codons analysed, 781 (99.87%) and 1500 (98.81%) were concordant in PR and RT regions, respectively (Table 1). One (0.13%) and 18 (1.19%) DRM pairs were partially concordant in the PR and RT regions respectively. The only partially concordant DRM pair in the PR region was due to detection of a mixture by the ViroSeq™ kit. For the only partially concordant DRM pair in the PR region, the ViroSeq™ assay detected the wildtype consensus amino acid in addition to the drug-resistance conferring amino acid. Of the 18 amino acid mixtures identified in the RT region, ViroSeq™ detected more (13/18; 0.86%) mixtures, as compared to in-house assay (5/18; 0.33%). There were no discordant pairs detected in DRM codons. Phylogenetic analysis of *pol* gene sequences shows tight clustering of sample pairs, which further confirmed the high degree of concordance between the 2 assays (Fig. 2).

For non-DRMs codons, more than 99% concordance was observed in both PR (99.34%) and RT (99.62%) regions. For detection of amino acid mixtures for non-DRMs codons, ViroSeq™ performed well (22 PR; 0.58%, 28 RT; 0.29%) when compared to in-house assay (2 PR; 0.05%, 7 RT; 0.07%). Three non-DRMs discordant mutations were observed in PR (1; 0.03%) and RT (2; 0.02%) regions.

Table 1. Comparison of In-House and ViroSeq™ Assay for Drug Resistance Testing in Wild-Type and Drug-Resistant Strains of HIV-1 (n = 46 specimens)

Pol gene	DRM positions		Non-DRM positions	
	PR	RT	PR	RT
Concordant	781 (99.87 %)	1500 (98.81%)	3747 (99.34%)	9669 (99.62%)
Partial concordant	1	18	24	35
- VS only	1 (0.13%)	13 (0.86%)	22 (0.58%)	28 (0.29%)
- IH only	0	5 (0.33%)	2 (0.05%)	7 (0.07%)
Discordant	0	0	1 (0.03%)	2 (0.02%)
Total codons analysed	782	1518	3772	9706

DRM: drug resistance mutation; IH: in-house (HIV-1 genotyping assay) PR: protease region; RT: reverse-transcriptase region; VS: ViroSeq™ (HIV-1 genotyping assay)
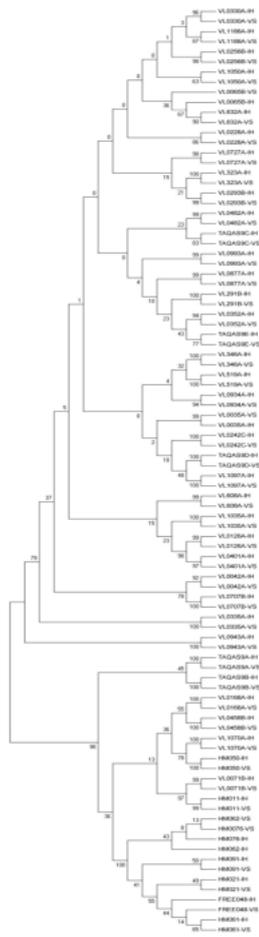
Fig. 2. Phylogenetic analysis of *pol* gene sequences from both in-house and ViroSeq™ assays.

Table 2. Performance of In-House and ViroSeq™ Assay in Detecting Amino Acids Conferring Drug Resistance Only (n = 46 specimens)

Pol gene	Amino acids conferring drug resistance only	
	PR	RT
Concordant	782 (100 %)	1505 (99.14%)
Partial concordant	0	13
- VS only	0	9 (0.60%)
- IH only	0	4 (0.26%)
Discordant	0	0
Total codons analysed	782	1518

IH: in-house (HIV-1 genotyping assay); PR: protease region; RT: reverse-transcriptase region; VS: ViroSeq™ (HIV-1 genotyping assay)

Table 2 shows the performance of in-house assay in the detection of amino acids conferring drug resistance only. From the results, there were 100% concordance in PR region and 99.14% in RT region. Of the 1518 DRM codons analysed in the RT region, 13 (0.86%) amino acid mixtures were partial concordance. Among the 13 amino acid mixtures, ViroSeq™ detected 9 (0.6%), while in-house detected 4 (0.26%). Details of comparison in detection of amino acid mixtures between ViroSeq™ and in-house assays are summarised in Table 3. The 13 DRMs positions analysed were derived from 8 patient samples, with only 1 partial concordant DRM identified in the PR region. In 9 of the 13 samples (92%), ViroSeqT™ detected mixtures of wild-type and mutant amino acids while in-house GRT assay only detected wild-type residue at positions associated with drug resistance. Further analysis of the 13 partial concordant

Table 3. Amino acid/ Base Substitutions at Drug Resistance Positions in Partially Concordant Mutations Detected by Both In-House and ViroSeq™ Assay

Category	Region	Codon	Amino acid mixtures detected by both assays			
			ViroSeq™		In-house	
			Amino acid	Base	Amino acid	Base
Partial concordance	PR	54	IV	RTC	V	GTC
	RT	65	K	AAA	KR	ARA
		67	ND	RAC	N/D/S	RRC
		69	TN	AMC	T	ACC
		69	TA	RCT	T	ACT
		75	I/V/M	RTR	M	ATG
		98	A	GCA	AS	KCA
		103	N	CAA	NK	MAA
		106	IV	RTY	V	GTT
		179	IV	RTT	V	GTT
		184	MV	RTG	V	GTG
		190	AG	GSA	A	GCA
		238	KR	ARA	K	AGA

PR: protease region; RT: reverse-transcriptase region

DRMs was carried out by comparing the alignment results between the 2 assays with drug resistance interpretation generated from Stanford HIV Drug Resistance Database. The 100% concordance of the drug resistance interpretations between the 2 assays had demonstrated the capability of in-house GRT assay in identifying and interpreting all clinically important DRMs.

The analysis of DRM patterns with its corresponding treatment regimen was based on the comparison between drug resistance interpretation results and available treatment history record. In a total of 33 subjects, the latest regimen when GRT was performed for 23 (70%) subjects were on a combination of NRTI and PI or NNRTI and PI; 10 (30%) subjects were on a combination of NNRTI and NRTI. None of the subjects was treated with a combination of NNRTI, NRTI and PI when GRT was performed.

Initial Experience with Implementing the In-House GRT for Clinical Use

The GRT assay has successfully amplified and sequenced 139 patient samples from the period of June 2011 to July 2012. Among the 139 patient samples successfully sequenced, there were 6 patient samples with viral load of between 175 to 485 copies/mL, 7 patient samples with viral load of between 501 to 999 copies/mL, while the remaining samples had viral load of between 999 copies/mL to 1,000,000 copies/mL.

In our experience of routine testing using the in-house GRT assay, there was also a small subset of patient samples that had DRM patterns unexpected of treatment regimen. We investigated further by subjecting all those samples for re-testing and verification by using the ViroSeq™ assay. No discrepancy in the DRM patterns reported by both the in-house and ViroSeq™ assays was found in all those samples re-tested.

The in-house GRT assay has performed satisfactorily in the 2 most recent proficiency testing panels (HIVG425 [17 November 2011] and HIVG425 [4 April 2012]) from the Treat-Asia Quality Assurance Scheme under the programme HIV-1 Genotypic Drug Resistance External Quality Assessment Scheme. In the 2011 proficiency-testing panel, detection of DRMs by the in-house GRT assay was 96% against the average performance of 96% from 35 participants (performance range: 84% to 100%). Detection of DRMs by the in-house GRT assay for the 2012 panel was 100% against the average performance of 94% from 30 participants (performance range: 79% to 100%).

Discussion

HIV-1 genotypic drug resistance testing is a standard of care in developed settings.[13] Access to commercial assays for drug resistance monitoring in Singapore is limited due to their high running cost. In the present study, we have evaluated the clinical performance of an in-house developed GRT assay. The results of our in-house assay demonstrated excellent correlation with the corresponding results from 46 samples validated against the FDA-approved commercial kit, ViroSeq™ assay.

Similar to the ViroSeq™ genotyping system, all 46 clinical samples with viral load of 500 copies/mL or greater were successfully amplified at the *pol* region in a reproducible manner, demonstrating the high sensitivity and reproducibility of the in-house primers. Notably, 5 samples among the 46 samples were from a panel of proficiency testing samples under TAQAS9 (2010) from the Treat-Asia HIV-1 Genotypic Drug Resistance External Quality Assessment Scheme.

The high concordance of drug-resistance conferring mutations (>99%) detected by the in-house GRT compared to ViroSeq™ system showed that both assays performed equally well in identifying clinically relevant mutations. The rate of partial discordance between the 2 assays was identified to be less than 1.0% and could be due to differential detection of nucleotide mixture in the samples.

High degree of genetic diversity characteristic of HIV-1 has been shown to hamper detection and accuracy of quantification of HIV genotyping assays.[8-10] Results obtained from the in-house GRT assays showed that the assay could detect resistance-conferring mutations in Singapore with no discernible difference when compared to results obtained from the ViroSeq™ system. No evidence of sample cross-contamination was observed, as contamination was minimised through the use of single non-nested RT-PCR for amplification.

Frequently, the high cost of commercial GRT curtails the establishment of an HIV drug resistance monitoring service in resource-limited settings. With projected running cost at SGD150 per test, the total reagents and consumables cost incurred for in-house assay would be less than half that of the ViroSeq™ assay (SGD150 vs SGD350). Such decrease in financial burden bearable by the patients will enable greater affordability to HIV drug resistance testing and hence, a wider coverage of the test within local HIV patients.

In routine practice, the in-house GRT assay has successfully sequenced a number of low viral load patient specimens (150 copies to <500 copies/mL) for genotypic resistance. Generally, resistance testing is not recommended when the viral load falls below 1000 copies/mL due to its lack of reliability in routine clinical applications.[18] On the other hand, several laboratories have experienced success in

genotyping clinical specimens at low HIV-1 viremia.[19-21] We are investigating further the clinical utility of the in-house GRT assay on low HIV-1 viremia samples.

Several theoretical limitations are important to keep in mind for the future improvement of the assay validation. A wider spectrum of viral loads and known quantified resistant mutants would facilitate accurate estimation of analytical detection limit of the genotyping assay. Nevertheless, all major PR and RT drug-resistance associated mutations were represented in the 46 validation samples currently presented. Additional ongoing effort to improve the in-house assay include comparison using a wider panel of HIV-1 subtypes and CRFs, as well as development of genotyping assays that include sequence regions beyond that of current methods.

Through this study, we verified that the clinical performance of the in-house assay is comparable to that of the commercial, FDA-approved commercial system, ViroSeq™. Our findings suggest that the in-house assay performs well in Singapore, as well as on international quality assurance testing. Our findings support the continued implementation of this low cost in-house assay for patient care.

REFERENCES

1. Vandamme AM, Camacho RJ, Ceccherini-Silberstein F, de Luca A, Palmisano L, Paraskevis D. European recommendations for the clinical use of HIV drug resistance testing: 2011 update. AIDS Rev 2011;13:77-108.

2. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. Available at: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed 24 August 2012.

3. Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, et al. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. JAMA 2012;308:387-402.

4. Dunn DT, Coughlin K, Cane PA. Genotypic resistance testing in routine clinical care. Curr Opin HIV AIDS 2011;6:251-7.

5. Johnson VA, Calvez V, Günthard HF, Paredes R, Pillay D, Shafer R, et al. 2011 update of the drug resistance mutations in HIV-1. Top Antivir Med 2011;19:156-64.

6. Kuritzkes DR, Grant RM, Feorino P, Griswold M, Hoover M, Young R, et al. Performance characteristics of the TRUGENE HIV-1 Genotyping Kit and the Opengene DNA Sequencing System. J Clin Microbiol 2003;41:1594-9.

7. Eshleman SH, Hackett J Jr, Swanson P, Cunningham SP, Drews B, Brennan C, et al. Performance of the Celera Diagnostics ViroSeq HIV-1 Genotyping System for sequence-based analysis of diverse human immunodeficiency virus type 1 strains. J Clin Microbiol 2004;42:2711-7.

8. Fontaine E, Riva C, Peeters M, Schmit JC, Delaporte E, Van Laethem K, et al. Evaluation of two commercial kits for the detection of genotypic drug resistance on a panel of HIV type 1 subtypes A through J. J Acquir Immune Defic Syndr 2001;28:254-8.

9. Mracna M, Becker-Pergola G, Dileanis J, Guay LA, Cunningham S, Jackson JB, et al. Performance of Applied Biosystems ViroSeq HIV-1 Genotyping System for sequence-based analysis of non-subtype B human immunodeficiency virus type 1 from Uganda. J Clin Microbiol 2001;39:4323-7.

10. Beddows S, Galpin S, Kazmi SH, Ashraf A, Johargy A, Frater AJ, et al. Performance of two commercially available sequence-based HIV-1 genotyping systems for the detection of drug resistance against HIV type 1 group M subtypes. J Med Virol 2003;70:337-42.

11. Ng OT, Munshaw S, Lamers SL, Chew KK, Lin L, Redd AD, et al. Molecular epidemiology of HIV type 1 in Singapore and identification of novel CRF01_AE/B recombinant forms. AIDS Res Hum Retroviruses 2011;27:1135-7.

12. Ng OT, Eyzaguirre LM, Carr JK, Chew KK, Lin L, Chua A, et al. Identification of new CRF51_01B in Singapore using full genome analysis of three HIV type 1 isolates. AIDS Res Hum Retroviruses 2012;28:527-30.

13. Hirsch MS, Brun-Vézinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, et al. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. JAMA 2000;283:2417-26.

14. Staden R, Beal KF, Bonfield JK. The Staden package, 1998. Methods Mol Biol 2000;132:115-30.

15. Saravanan S, Vidya M, Balakrishnan P, Kumarasamy N, Solomon SS, Solomon S, et al. Evaluation of two human immunodeficiency virus-1 genotyping systems: ViroSeq 2.0 and an in-house method. J Virol Methods 2009;159:211-6.

16. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.

17. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 2004;101:11030-5.

18. Hammer SM, Eron JJ Jr, Reiss P, Schooley RT, Thompson MA, Walmsley S, et al. Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. JAMA 2008;300:555-70.

19. Gale HB, Kan VL, Shinol RC. Performance of the TruGene human immunodeficiency virus type 1 genotyping kit and OpenGene DNA sequencing system on clinical samples diluted to approximately 100 copies per milliliter. Clin Vaccine Immunol 2006;13:235-8.

20. Waters L, Mandalia S, Asboe D. Successful use of genotypic resistance testing in HIV-1-infected individuals with detectable viraemia between 50 and 1000 copies/ml. AIDS 2006;20:778-9.

21. Yang C, McNulty A, Diallo K, Zhang J, Titanji B, Kassim S, et al. Development and application of a broadly sensitive dried-blood-spot-based genotyping assay for global surveillance of HIV-1 drug resistance. J Clin Microbiol 2010;48:3158-64.