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Drug resistance mutations among virological failure HIV-1 infected patients in Malaysia

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Abstract. The determination of HIV drug resistance mutations (DRMs) towards antiretroviral (ARV) drugs among HIV-1 treated patients with virological failure is crucial for further management of the patient. This study aimed to assess the most common genomic mutation and to analyse subtypes among the HIV-1 patients with viral load level ≥ 1,000 copies/mL. A total of 101 virological failure HIV-1 patients from four different regions of Peninsular Malaysia with a viral load measurement facility were included in the study. Majority of patients (89.1%) have at least 1 mutation associated with clinical resistance to either protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) or nonnucleoside reverse transcriptase inhibitors (NNRTIs). Major resistance mutations among the patients towards NRTIs and NNRTIs were 70.3% and 18.8%, respectively. The most common mutation for NRTIs was M184V while K103N mutation was detected in the majority of patients who were treated with NNRTIs. The most commonly observed mutations for major PI and minor PI seen among the study population were V82A/T and L10V, respectively. In HIV-1 subtype analysis, CRF33_01B was the most predominant HIV-1 subtype in this study group. The vast detection of DRMs in this study emphasized the importance of genotypic resistance test in the management of HIV patients as DRMs can alter patient's susceptibility towards ARV drugs. Further study on larger number of samples is essential for the development of a database on HIV-1 DRMs among patients that experience virological failure in Malaysia.

INTRODUCTION

Since the introduction of Highly Active Antiretroviral Therapy (HAART) in 2006 for Human Immunodeficiency Virus Type 1 (HIV-1) treatment by Ministry of Health Malaysia, the determination of drug resistance mutations (DRMs) to antiretroviral (ARV) drugs among the HIV population was carried out only at selected facilities. This test was not available to all HIV-1 patients due to its limited access and resources. Therefore, only limited data was available on DRMs even though the HIV-1 genotyping assay has been made available in Malaysia for more than a decade.

The use of ARV drugs is still the mainstay of treatment for HIV-1 patients worldwide including Malaysia. Up till December 2014, there were a total of 17,340 people living with HIV who were on treatment in Malaysia (Sha'ari, 2015). The presence of six major groups of ARV drugs with more than 20 drugs available in the market, the suppression of HIV-1 replication to undetectable level can be achieved as well as an improvement of CD4 count and a reduction of opportunistic infection can be clearly seen among HIV-1 treated patients. In Malaysia, with the introduction of more affordable and accessible first and second line ARV drugs, the number of AIDS-related deaths has

decreased where only 756 AIDS-related deaths cases were reported in 2014 (Sha'ari, 2015).

However, HIV-1 is notorius for its high replication, mutation and recombination rates which can lead to the production of many drug-resistant mutants (Gianella, Richman, 2010), leading to treatment failure. As a consequence, there is a need for more expensive second and third regimes to treat these patients. This is one of the major hurdles that need to be faced by the clinician in Malaysia. Apart from WHO consolidated guidelines on the use of ARV drugs for treating and preventing HIV infection, Ministry of Health Malaysia has developed a local third edition guidelines for the management of adult HIV in 2011 which provides details on the judicious use of ARV agents based on local accessibility and price considerations.

The overall level of nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) resistance among the virological failure was reported between 52 to 92% and 43 to 100%, respectively by WHO Southeast Asia Region (SEAR) (Trotter *et al*., 2013). Meanwhile, a systematic review of HIV drug resistance within SEAR generally showed a low level frequency of Protease Inhibitor (PI) resistance with a range of 0.0 – 10.6% in patients failing PI-based first-line regime of ARV drugs (Trotter, *et al*., 2013). In addition to the previous reports on DRMs in certain parts of Malaysia, this data can further assist and guide the physicians to select the most effective and appropriate ARV therapy regime for HIV-1-infected patients who have developed resistance towards the drug treatment. Hence, this study is targeting to determine the most common HIV-1 genomic mutation region(s) and subtype(s) among HIV-1 patients with virological failure in our country.

MATERIALS AND METHODOLOGY

From January 2012 to December 2013, a total of 111 frozen plasma samples were collected from four different centres performing viral load quantitation which include Sultanah Aminah Hospital, Johor Bharu; Sg Buloh Hospital, Selangor, Pulau Pinang Hospital, Pulau Pinang & Raja Perempuan Zainah II Hospital, Kota Bharu, Kelantan (representing the Southern, Central, Northern and Eastern regions) in Peninsular Malaysia. 5 ml of blood samples from HAART-experienced patients were collected in ethylenediaminetetraacetic acid (EDTA) blood collection tubes. Plasma was separated from the collected blood within 2 hours of collection by centrifugation at 2000 rpm for 10 minutes and stored at -80° C prior to transportation to our laboratory. All samples were transported in containers with dry ice or ice packs. Upon arrival at our laboratory, the samples were immediately stored at -80°C until further testing is done. The study was approved by the Medical Research and Ethical Committee of the Malaysian Ministry of Health with identification number NMRR-11-181-8832 and Universiti Kebangsaan Malaysia (UKM) Ethical Committee with a project code FF-2014-339. Informed consents were obtained prior to samples collection. All HIV-1 adult patients who had received antiretroviral therapy (ART) and had shown virological failure with HIV-1 RNA levels of $\geq$ 1,000 copies/mL were included in the study.

Prior to extraction of RNA, the samples were centrifuged at 20,800 g at 4°C for 75 minutes. Viral RNA was extracted from 1ml of plasma by column purification method (QIAamp Viral RNA Mini Kit, Qiagen – Germany), according to manufacturer's instructions. The samples were analyzed for HIV-1 drug resistance mutations involving the entire protease and two-third of reverse transcriptase of the *pol* gene. The extracted RNA was reverse transcribed by using the primer RT21 (5'-CTGTATTTCTGCTATTAA GTCTTTTGATGGG-3') with the following reactions: 65°C for 5 min, 4°C for 2 min, 50°C for 60 min, 85°C for 5 min, 4°C for 6 min, 37°C for 20 min, 4°C for 5 mins. The first round PCR was performed by using primers MAW26 (5'-TTGGAAATGTGGAAAGGAAGGAC-3') and RT21 (5'- CTGTATTTCTGCTATTAAGTC TTTTGATGGG-3') with the following steps: 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 56°C for 45 sec and 72°C for 90

sec, with a final extension step of 5 min at 72°C. The PCR products were then subjected to nested PCR by using primers PRO1 (5'-CAGAGCCAACAGCCCCACCA- 3') and RT21 (5'- CTGTATTTCTGCTATTAAGTCTT TTGATGGG-3') with the following cycling conditions: a denaturation step of 3 min at 95°C, and 40 cycles of 30 sec at 95°C, 45 sec at 60°C and 90 sec at 72°C, with a final extension at 72°C for 1 min.

The PCR products were analyzed on 1.8% SYBR® safe DNA stained agarose electrophoresis gel. The resulting PCR-amplified DNA products with expected size of 1300-1400 bp were further purified by using QiaQuick gel extraction kit. The purified DNA products were subjected to cycle sequencing by using ABI PRISM® BigDye® Terminator v3.1 cycle sequencing kit with the following reactions: 1 cycle of 96°C for 1 min, 40 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, 1 cycle of 4°C for 5 min. Subsequently, the nucleotide sequences of protease (PR) and two third of reverse transcriptase (RT) were determined by cycle-sequencing dideoxy chain termination method on an automated DNA sequencer (ABI PRISM 3730 Genetic Analyzer, Applied Biosystems – USA) by using four forward and three reverse primers as shown in Table 1.

Nucleotide sequences of the PR and 2/3 of RT were edited, aligned and analyzed by using ChromasPro version 1.5 software with HXB2 sequence of HIV-1 as a reference sequence. The fasta format for each individual sample's sequence was generated and deposited in the Stanford HIV Resistance Database (http://hivdb.stanford.edu/) for the analysis of HIV drug resistance mutations. HIV-1 subtype analysis was done by using NCBI Genotyping Tools (reference sequence set 2009). The nucleotide sequences also were aligned by using Clustal W in the MEGA 6 version 6.0.5 program. Phylogenetic analysis was performed by using the neighbour-joining (NJ) method (Tamura, *et al*., 2013).

The performance of in-house HIV-1 drug resistance assay was assessed through participation in the TREAT Asia Quality Assurance Scheme (TAQAS), which was organised by the National Serology Reference Laboratory, Australia. Three testing panels with 15 proficiency testing samples with different viral load levels were analyzed by the assay. The analysis of the sequences was performed by the organizer based on the consensus target genotypes that were generated from the participants' results.

RESULTS

Of the 111 samples received, 101 were successfully amplified and sequenced for the PR and RT genes. 100 samples had viral load levels 1,000 copies/ml with the mean HIV-1 viral load of $\log_{10}$ 5.15 copies/ml and a median of $\log_{10}$ 4.61. One sample had unknown viral load level. Fifty four (53.5%) samples belonged to male patients and forty seven (46.5%) samples were from female patients. In this study population, seventy (69.3%) patients were Malay, seventeen (16.8%) patients were Chinese and thirteen (12.9%) patients were Indian and one patient

Table 1. Primers for the cycle sequencing of the HIV drug resistance genotyping assay

Primers	Sequence 5' to 3'	HXB2 Location (nt)
Forward	CAGAGCCAACAGCCCCACCA	2147-2166
	GTTGACTCAGATTGGTTGCAC	2519-2539
	TAAAATTAAAGCCAGGAATGGATG	2578-2601
	GGATGGAAAGGATCACC	3003-3019
Reverse	CTGTATTTCTGCTATTAAGTCTTTTGATGGG	3539-3509
	ATGCCTTTATTTTTTCTTCTGTC	2649-2627
	GGTGATCCTTTCCATCC	3019-3003

belonged to other ethnicity. The participant age ranged from 18 to 72 years with the median age of 38 years. Proportion of samples received was forty three (43), twenty three (23), nine (9) and twenty six (26) samples from Eastern, Southern, North and Central areas of Peninsular Malaysia, respectively. The entire study participants were either on HAART or had previous exposure to HAART. Distribution of patients who were on NRTIs and NNRTIs, NRTIs and PIs, and NRTIs, NNRTIs and PIs was 75% (76/101), 10.9% (11/101) and 3.0% (3/101), respectively. The ARV used for 9 patients who were on the treatment during the study period could not be retrieved. Meanwhile in 2 patients, only PIs were used in their treatment.

The HIV-1 subtypes were determined using NCBI Genotyping system based on HIV-1 2009 reference set. Fifty four (53.4%) of patients were infected with HIV-1 CRF 33_01B while thirty three patients (32.7%) were found to have HIV-1 subtype CRF01_AE. Other HIV-1 subtypes detected were HIV-1 subtype B (10, 9.9%), CRF15_01B (3, 3.0%) and CRF02_AG (1, 1.0%).

Among 101 HIV-1 patients with virological failure, 90 (89.1%) of them have at least 1 mutation to either PI, NRTIs or NNRTIs. Two (2.2%) of these patients had major resistance mutations associated with PIs which had a reduced susceptibility towards all drugs within the group except darunavir/r (DRV/r). Of these two patients, one also had resistance towards NRTIs and NNRTIs. Meanwhile, minor mutations towards PI were detected in 23/90 (25.6%) of patients and a mutation at codon 10 (L10V) was the most common mutation detected. Five (21.7%) of these 23 patients had mutations towards PI alone.

Major resistance mutations towards NRTIs and NNRTIs occurred in 71/101 (70.3%) and 19/101 (18.8%) patients, respectively. Among 49 drug resistance mutations (DRMs), M184V was found to be the most frequent mutation found for NRTIs while out of 46 DRMs identified, K103N mutation was detected in the majority of patients who were treated with NNRTIs. Details of DRMs are shown in Table 2. Meanwhile, 11/101 (10.9%) patients did not show any mutations.

Subtype analysis, based on protease and reverse transcriptase regions showed that CRF33_01B (45/101, 44.6%) was the most predominant HIV-1 subtype in this study group. HIV-1 subtype CRF01_AE was found in 39 patients, subtype B in 12 patients, subtype CRF15_01B in 4 patients and subtype CRF02_AG in 1 patient. However, in Southern and Northern part of Peninsular Malaysia, CRF01_AE was found to be the most frequent HIV-1 subtype seen while in Eastern and Central part of Peninsular Malaysia, it was CRF33_01B subtype. Phylogenetic analysis of the subtypes is shown in Figure 1.

The performance of this in-house genotyping assay was evaluated by the annual TREAT Asia Quality Assessment Scheme (TAQAS) programme 2013 to 2014. The results had demonstrated >95% agreement at the nucleotide sequence and detection of DRMs level with other participants.

DISCUSSION

This report demonstrates 89.1% of HIV-1 patients with virological failure in Malaysia harboured at least one of DRMs to NRTIs, NNRTIs and PIs. Similar high prevalence rate (77.8%) was previously reported in 2006 by Malaysian Researchers who studied the antiretroviral-treated HIV-1 patients with suboptimal virological response. However, all participants were only from University Malaya Medical Centre, Kuala Lumpur (Tee *et al*., 2006). The current study also showed that the majority of the patients were on both NRTIs and NNRTIs as they are the two major groups of antiretroviral (ARV) drugs used in Malaysia in treating HIV infection. The finding of 95 DRMs for NRTIs and NNRTIs as compared to only 15 DRMs for PIs is also comparable to previous report on DRMs towards ARV in Malaysia (Tee *et al*., 2006). Despite the availability of the guidelines on the ARV therapy; two patients were treated with PIs alone, and further history on the treatment could not be clarified.

Table 2. Details of major drug resistance mutations identified

PI Minor			PI Major		
DRMs Detected [Σ = 11]	Frequency	%	DRMs Detected [Σ = 4]	Frequency	%
A71V/AT	4	16.0	I54IV	1	25
K20M/IK	2	8.0	M46I	1	25
K43KT	1	4.0	V82A/T	2	50
L33F	2	8.0			
L10I/V/IL/LV	15	60			
V11IV	1	4.0			
NRTI			**NNRTI**		
DRMs Detected [Σ = 48]	Frequency	%	DRMs Detected [Σ = 47]	Frequency	%
A62V	1	0.6	Y181C/CY/I	23	12.8
D67G/N/DG/DN	16	9.1	G190AG/S/A/C	15	8.4
E138Q	1	0.6	V179D/T/L/DV/IT/T	12	6.7
F116Y	1	0.6	Y188L/FHLY	8	4.5
K65R/KR	6	3.4	K103N/KT/NS/KN	43	24.0
K70G/R/EQ/KQ/KR	15	8.5	P225H	11	6.1
K103N	1	0.6	K101E/EK/P/H/N/EK	14	7.8
K219E/Q/R/KQ	11	6.3	E138Q/G/AE	6	3.4
K238T	1	0.6	K238T	3	1.7
L74I/V/IL/IV/LV	7	4.0	A98G/AG	5	2.8
L210W/LW	4	2.3	V90I	5	2.8
M41L/LM	16	9.1	V106IV/A/I/M	9	5.0
M184V/I/MV	56	31.8	H221HY/Y	10	5.6
Q151M/KLMQ	2	1.1	F227L	2	1.1
T69I/N/S/NT	11	6.3	L100I	3	1.7
T215F/S/Y/FY/IT	18	10.2	V108IV/V/I	5	2.8
T115F	1	0.6	M230L/LM	4	2.2
V75I/M/LV	6	3.4	Y318F	1	0.6
Y115FY	2	1.1			

In this study, M184V which is located in RT's conserved area close to the active site frequently emerges during the treatment with lamivudine, emtricitabine, abacavir and less commonly with didanosine (Karidia *et al*., 2003). This was the most commonly occurring NRTI mutation observed. The study demonstrated that this mutation alone has contributed about 31.8% (56/176) of all DRMs to NRTIs. Meanwhile for NNRTIs, 23.7% (43/181) of all mutations were K103N/KT/NS/KN mutations with K103N, a nonpolymorphic mutation selected in patients receiving Nevirapine (NVP) and Efavirenz (EFV). It was found to be the most common mutation for NNRTI in this study. This was followed by Y181C, 11.0% (20/181).

Major PI mutations detected in this study were M46I, I54IV and V82A/T. The occurrence of M46I, I54IV and V82A mutations in a patient who was on NRTIs (zidovudine, didanosine and lamivudine) and PI (lopinavir) might be due to the presence of PI pressure. However, the duration of the treatment and the patient's previous treatment history could not be obtained. Meanwhile, V82T was seen in another patient whose the treatment history could not be retrieved and probably the natural sequence variant. Another limitation in this study, apart from the small sample size,

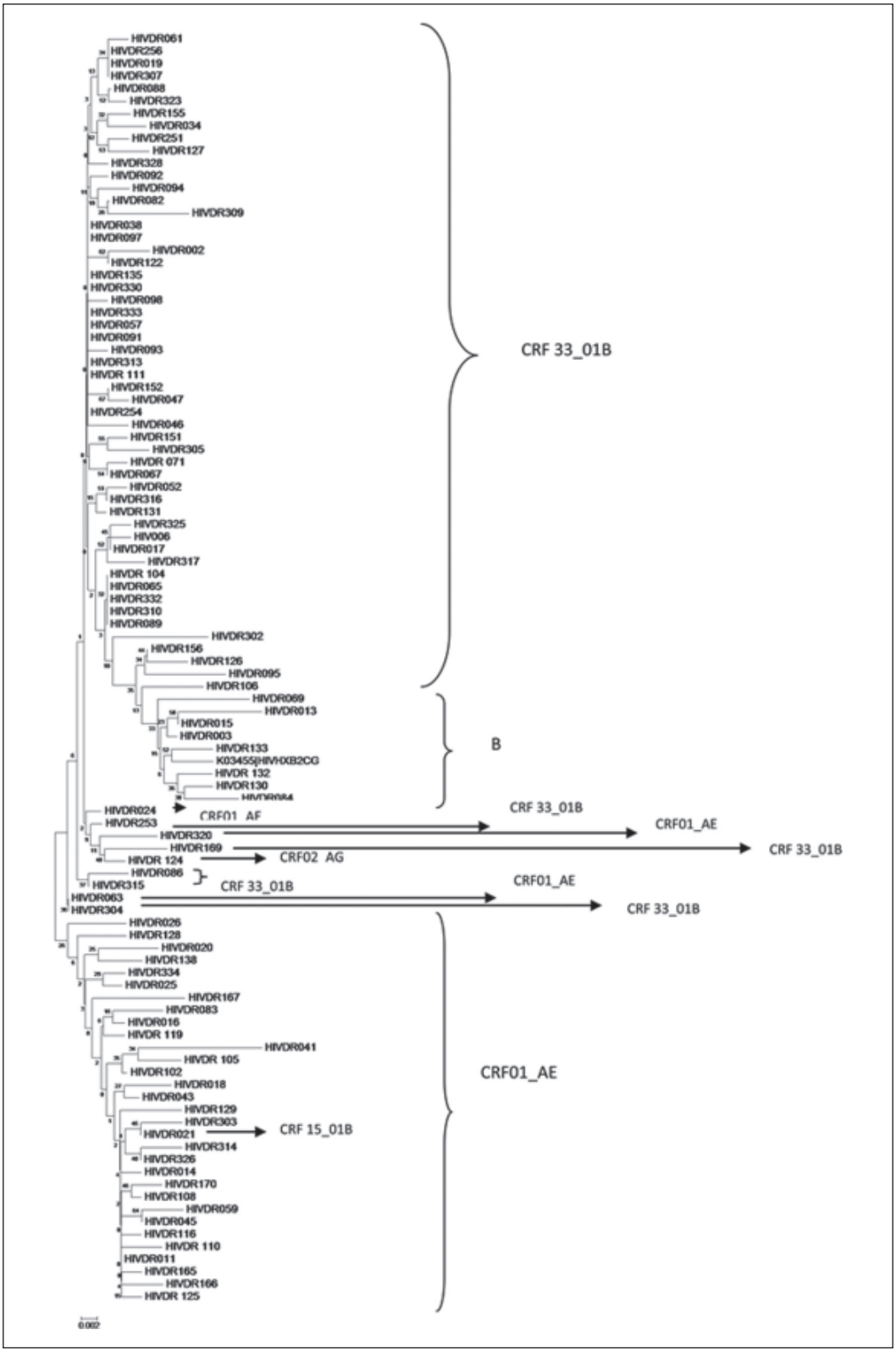

Figure 1. Phylogenetic tree of all samples tested and showed the predominant circulating HIV-1 subtype is CRF33_01B. It is shown also 8 samples belonged to outlier.

was the absence of treatment duration for each individual patient which might add further value to the significance of all DRMs reported. In this study, eleven patients with evidence of virological failure did not show any mutations towards NRTIs and NNRTIs which could be due to patient-related medication compliance.

Currently, the common HIV-1 subtypes reported in Malaysia are HIV-1 subtype B, CRF01_AE and CRF33_01B (Tee *et al*., 2006, Chook, *et al*., 2015). As illustrated in Figure 2, CRF33_01B was found to be the major circulating HIV-1 subtype in this population and it was dominant in Eastern and Central regions of Peninsular Malaysia. Similar finding was demonstrated by the study of 30 HIV-1 treated children who failed ARV therapy in East Coast of Peninsular Malaysia (Mohamad *et al*., 2012, Ariffin *et al*., 2014) and this subtype was detected at high prevalence among urban population in Kuala Lumpur (Chook, *et al*., 2015). CRF33_01B, a unique recombinant form which composed of CRF01_AE and subtype B was first reported in Malaysia with high prevalence among intravenous drug users in 2006 (Chook, *et al*., 2015). Apart from this subtype, a number of CRFs were reported to be circulating in Malaysia including the newly described CRFs: CRF53_01B, CRF54_01B, and CRF58_01B (Chook, *et al*., 2015).

Based on the phylogenetic tree of HIV-1 subtypes of the study population, there were 3 different major groups identified: CRF33_01B, CRF01_AE and B. Only one sample was identified as an A2_AG subtype. Eight other sequences were outliers even though they were labelled as either CRF33_01B or CRF01_AE and appeared as one different cluster. All these samples need further analysis to illustrate better relatedness among all subtypes.

Geographical distribution of different HIV-1 subtype and drug resistance mutations' pattern warrants further assessment as no data on the HIV-1 subtype was available for certain areas of Malaysia, including Sabah and Sarawak. Furthermore, most of HIV drug resistance studies were mainly performed in Kuala Lumpur and Kelantan due to the availability of research facilities in these 2 locations. The data from current study was also very limited due to its small sample size and the proportion of sample collected from the specified areas was not equal. Thus, more samples need to be collected and analysed from these regions, which could be limited by logistic reasons. It is also important to assess dried blood spot from various states in Peninsular Malaysia as an alternative sample in performing HIV-1 drug resistance testing. Small sample size for the study was inevitably due to the limited budget for the study.

CONCLUSION

We report the overall pattern of drug resistance mutations among HIV-1 patients with virological failure and the HIV-1 subtypes in Peninsular Malaysia. This report involving different regions of Peninsular Malaysia can provide further information on HIV drug resistance which can serve as a foundation for HIV surveillance data development for the country. More data on DRMs can be accumulated with the availability of more HIV drug resistance genotyping assay laboratories in the country. This can further contribute to HIV-1 drug resistance data in Asia.

Sequence Data

Nucleotide sequences of RT and protease gene were submitted to GenBank under accession number KT281488-KT281587.

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