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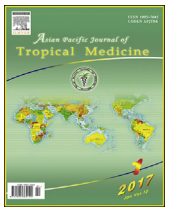

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Identifications of drug resistance mutations among antiretroviral treatment naive HIV-1 patients in Peninsular Malaysia

Rozainanee Mohd Zain✉, Nabila Ibrahim, Suriani Ismail, Jeyanthi Suppiah, Nor Aziyah Mat Rahim, Ravindran Thayan

Virology Unit, Institute for Medical Research, Infectious Disease Research Centre, Jalan Pahang, 50588, Kuala Lumpur, Malaysia

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ABSTRACT

Objective: To determine drug resistance mutations and the HIV-1 subtypes among antiretroviral treatment naive HIV-1 patients in Peninsular Malaysia.
Methods: A total of 45 samples from four hospitals that provide HIV viral load services were subjected to the amplification of the protease and two third of reverse transcriptase regions of the *pol* gene by RT-PCR and Sanger sequencing. Drug resistance mutation (DRM) interpretation reports the presence of mutations related to protease inhibitors (PIs), Nucleoside reverse-transcriptase inhibitors (NRTI) and Non-nucleoside reverse-transcriptase inhibitors (NNRTI) based on analysis using Stanford HIV database program.
Results: DRMs were identified in 35% of patients, among which 46.7% of them showed minor resistance to protease inhibitor with A71V and L10I were the commonest DRMs detected. About 21.4% and 50.0% of patients had mutations to NRTIs and NNRTIs, respectively. CRF01_AE was found to be the predominant HIV-1 subtype.
Conclusions: These findings have served as an initial crucial data in determining the prevalence of transmitted HIV-1 drug resistance for the country. However, more samples from various parts of the country need to be accumulated and analyzed to provide overall HIV-1 drug resistance in the country.

1. Introduction

Till 31st December 2014, Malaysia had 88 093 cases of people living with HIV and 3517 new HIV cases were reported to the Ministry of Health in 2014 [1]. Even though Malaysia is one of the countries with more than 5% of HIV infection rate among people who inject drugs, sex workers, transgender and men having sex with men population, a notification rate of new cases each year continues to decrease with only 11.7 cases per 100 000 population was reported in 2014 [1].

The Government of Malaysia has given full commitment in providing high standard management of clinical HIV in the hospital and primary healthcare systems. Besides, ensuring the safety of blood and blood products, measures have been taken to improve the treatment availability and to reduce treatment cost, especially those related to antiretroviral (ARV) drugs. Two major achievements in the country thus far are the availability of the first line ARV treatment to the patients as well as to the imprisoned populations in particular, the HIV positive prisoners and inmates in drug rehabilitation centres. Thus, by end of 2014, 21 481 people living with HIV (including 328 paediatric cases) are on ARV, where the majority of them were still on treatment after one year and virally suppressed with viral load less than 1 000 copies/mL [1].

Scaling up on the ARV treatment among HIV-1 patients may pose a significant impact on the HIV drug resistance if it is not carefully monitored. Moreover, failure to response to ARV drugs among HIV-1 naive patients could be attributed to transmitted HIV-1 drug resistance. This worrisome situation has been addressed by the WHO as part of the Global Action Plan for HIV drug resistance 2016–2021 in order for the 90-90-90 targets could be achieved by the 2020 [2,3]. The targets are aimed for 90% of PL HIV aware of their HIV status, 90% of those diagnosed will be treated with antiretroviral therapy (ART) and 90% of those complied with ART show a significant reduction in HIV-1 copies number with less than 1 000 copies/mL [2,3].

✉First and corresponding author: Rozainanee Mohd Zain, Virology Unit, Institute for Medical Research, Infectious Disease Research Centre, Jalan Pahang, 50588, Kuala Lumpur, Malaysia.
Tel: +60 3 26762671
Fax: +60 3 26938094
E-mail: rozainanee@yahoo.com

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The transmitted HIV-1 drug resistance is defined as the discovery of drug resistance mutation in patients who are newly diagnosed with HIV and not yet on any ARV treatment [4]. The prevalence of transmitted resistant among HIV-1 infected person is not readily reported in many South East Asia regions [4,11,12], including Malaysia. Current data that are available on transmitted HIV-1 resistance in Malaysia was confined only to Kuala Lumpur and Kelantan. Therefore, this study aimed to determine the drug resistance mutations among HIV-1 naive patients from Peninsular Malaysia and to analyse the current circulating HIV-1 subtype in this study population.

2. Materials and methods

2.1. Samples

A total of 45 samples were collected from HIV-1 adult patients who were newly diagnosed for HIV infection and had not received ART from 4 different viral load centres which to include Sultanah Aminah Hospital, Johor Bharu; Sg Buloh Hospital, Selangor, Pulau Pinang Hospital, Pulau Pinang & Raja Perempuan Zainab II Hospital, Kota Bharu, Kelantan (representing the Southern, Central, Northern and Eastern regions, respectively) in Peninsular Malaysia. The study protocol was approved by the Medical Research and Ethical Committee of the Malaysian Ministry of Health with identification number NMRR-11-181-8832. Prior to 5 mL of EDTA blood collection, an informed consent was obtained from the each patient. Plasma separation was performed by centrifuging the blood at 2 000 rpm for 10 min within 2 h of collection. Due to logistic reasons, the plasma was kept at −80 °C prior to transportation to our laboratory.

2.2. RNA isolation

Prior to extraction of RNA, all samples were subjected to centrifugation at 20 800 g at 4 °C for 75 min. Based on the instructions from the manufacturer, viral RNA was extracted from 1 ml of plasma by column purification method (QIAamp Viral RNA Mini Kit, Qiagen–Germany).

2.3. Protease and 2/3 reverse transcriptase amplification

The entire protease and two-third of reverse transcriptase of the *pol* gene were analyzed for the HIV drug resistance mutations. The extracted RNA was converted to cDNA by using the primer RT21 (5′-CTGTATTTCTGCTATTAAGTCTTTTGATGGG-3′) as the following: 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 min. Primers MAW26 (5′-TTGGAAATGTGGAAAGGAAGGAC-3′) and RT21 (5′-CTGTATTTCTGCTATTAAGTCTTTTGATGGG-3′) were used in the first round PCR with the following steps: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 90 s, with a final extension step of 5 min at 72 °C. Each amplicon was further subjected to nested PCR by using primers PRO1 (5′-CAGAGCCAACAGCCCCACCA-3′) and RT21 (5′-CTGTATTTCTGCTATTAAGTCTTTTGATGGG-3′) with the following cycling conditions: a denaturation step of 3 min at 95 °C, and 40 cycles of 30 s at 95 °C, 45 s at 60 °C and 90 s at 72 °C, with a final extension at 72 °C for 1 min.

2.4. Post PCR purification and sequencing

The PCR products were analyzed on 1.8% SYBR® safe DNA stained agarose electrophoresis gel. The expected size of amplicons were purified by QiaQuick gel extraction kit (Qiagen, Germany), then 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 s, 50 °C for 5 s and 60 °C for 4 min, 1 cycle of 4 °C for 5 min. Subsequently, the excess dye terminators were removed using DyeEx Spin kit (Qiagen, Germany), the nucleotide sequences of protease (PR) and two third of reverse transcriptase (RT) were analyzed on an automated DNA sequencer (ABI PRISM 3730 Genetic Analyzer, Applied Biosystems-USA) using primers as shown in Table 1.

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

Primers	Sequence 5′ to 3′	HXB2 Location (nt)
Forward	CAGAGCCAACA GCCCCACCA	2147–2166
	GTTGACTCAGA TTGGTTGCAC	2519–2539
	TAAAATTAAAGCCA GGAATGGATG	2578–2601
	GGATGGAAAGGATCACC	3003–3019
Reverse	CTGTATTTCTGCTAT TAAGTCTTTTGATGGG	3539–3509
	ATGCCTTTATTT TTTCTTCTGTC	2649–2627
	GGTGATCCTTTCCATCC	3019–3003

2.5. Data analysis

The nucleotide sequences of the entire protease and two-third of RT were assembled and aligned to the HXB2 consensus by using ChromasPro version 1.5 software. Any differences in base calling data were identified. HIV drug resistance analyses were performed using Stanford HIV Resistance Database (http://hivdb.stanford.edu/). Meanwhile all sequences were aligned using Clustal W algorithm (MEGA 6 v 6.0.5) and the phylogenetic analysis was inferred by using the neighbour-joining (NJ) method. The analysis was conducted in MEGA 6 [5]. NCBI Genotyping Tools (reference sequence set 2009) was used to perform the HIV-1 subtype analysis.

3. Results

3.1. HIV-1 drug resistance mutations

The protease and RT regions of *pol* gene were successfully amplified in 40 patients. The majority of samples received (25/40) was from Central region while only 3 samples were received from Northern area. Samples collected from Southern and Eastern regions were 11 and 1, respectively. A total of 37 study participants were male while the rest were female. Participants' age ranged from 19 to 60 years. Only 40.0% (16/40) of patients had a viral load level stated in the request form. The viral load value for 12 patients were traced from the respective hospital by taking the first viral load value that appeared in the system. However, no viral load value was observed for 12 patients. CD4 count was not available for all patients. The majority of patients (65.0%) did not have any DRMs to all antiretroviral drugs while

of 40 patients, 14 (35.0%) patients showed at least one mutation to at least one of the protease inhibitors (PIs), NRTIs and NNRTIs. 7/15 patients with DRMs showed minor resistance to PIs. A71V and L10I were found to be the commonest DRMs detected in this study population. While other mutations detected towards PIs were I84IM, L33F and K20I. None of the patients demonstrated major resistance towards PIs.

Out of 14 patients, 3 (21.4%) and 7 (50.0%) of patients had mutations to NRTIs and NNRTIs, respectively. One patient reported K65R, Y115F and M184V mutations while one patient each reported to have T69NT and K219R for the NRTIs. Details of the viral load level, HIV-1 resistance mutations and subtypes of each participant are shown in Table 2. Nucleotide sequences of RT and protease gene were submitted to Genbank with accession number KU894653–KU894693.

3.2. HIV subtyping

CRF01_AE (19/40) was found to be the main HIV-1 subtype among these patients. While CRF 33_01B and B subtypes were detected in 11 and 7 patients, respectively. Two patients were infected with HIV-1 subtype G and one patient was found to have HIV-1 subtype 34_01B.

Table 2
HIV-1 genotypes and drug resistant mutations analyses in ARV naive HIV-1 infected patients.

Sample ID	Viral (copies/mL)	Subtype	PI minor	NRTI resistant mutations	NNRTI resistant mutations
MYHIVDR001	5.25	CRF 33_01B	–	–	–
MYHIVDR004	5.43	CRF 33_01B	–	–	–
MYHIVDR005	5.03	CRF01_AE	–	–	V106I
MYHIVDR009	4.99	CRF01_AE	L10I	–	–
MYHIVDR023	4.96	CRF 33_01B	–	–	–
MYHIVDR028	5.22	CRF 33_01B	–	–	V179DV
MYHIVDR029	5.22	CRF01_AE	–	–	–
MYHIVDR030	5.11	CRF01_AE	–	–	–
MYHIVDR031	6.37	CRF 33_01B	–	–	–
MYHIVDR035	5.75	B	–	–	–
MYHIVDR042	4.75	CRF01_AE	L10I	–	–
MYHIVDR101	6.48	CRF01_AE	–	–	–
MYHIVDR103	–	B	–	–	–
MYHIVDR107	–	CRF01_AE	–	–	–
MYHIVDR112	–	CRF01_AE	–	–	–
MYHIVDR113	–	CRF01_AE	–	–	–
MYHIVDR114	6.48	CRF01_AE	–	–	–
MYHIVDR115	5.83	CRF01_AE	–	–	–
MYHIVDR139	–	B	–	–	V106I
MYHIVDR140	5.17	B	–	–	–
MYHIVDR141	–	CRF01_AE	–	–	–
MYHIVDR142	–	CRF 33_01B	–	–	–
MYHIVDR143	4.00	CRF01_AE	–	–	–
MYHIVDR144	4.72	G	–	–	V90I
MYHIVDR145	–	CRF01_AE	–	–	
MYHIVDR146	–	B	A71V	–	E138A
MYHIVDR147	–	CRF 33_01B	–	–	–
MYHIVDR148	3.70	CRF01_AE	–	–	–
MYHIVDR149	4.45	CRF01_AE	–	–	–
MYHIVDR150	5.01	CRF01_AE	I84IM	–	–
MYHIVDR157	4.60	B	A71V	–	–
MYHIVDR162	–	CRF01_AE	L33F	–	–
MYHIVDR168	–	G	K20I	–	V90I
MYHIVDR337	4.64		–	K65R, Y115F, M184V	K103N, Y188L
MYHIVDR351	2.98	B	–	–	–
MYHIVDR352	4.12	CRF 33_01B	–	–	–
MYHIVDR353	4.44	CRF 15_01B	–	–	–
MYHIVDR354	5.83	CRF 33_01B	–	T69NT	–
MYHIVDR355	5.40	CRF 34_01B	–	–	–
MYHIVDR356	4.59	CRF01_AE	–	K219R	–

3.3. Phylogenetic analysis

Genetic relatedness of each HIV-1 strain was performed and it was shown in phylogenetic tree in Figure 1.

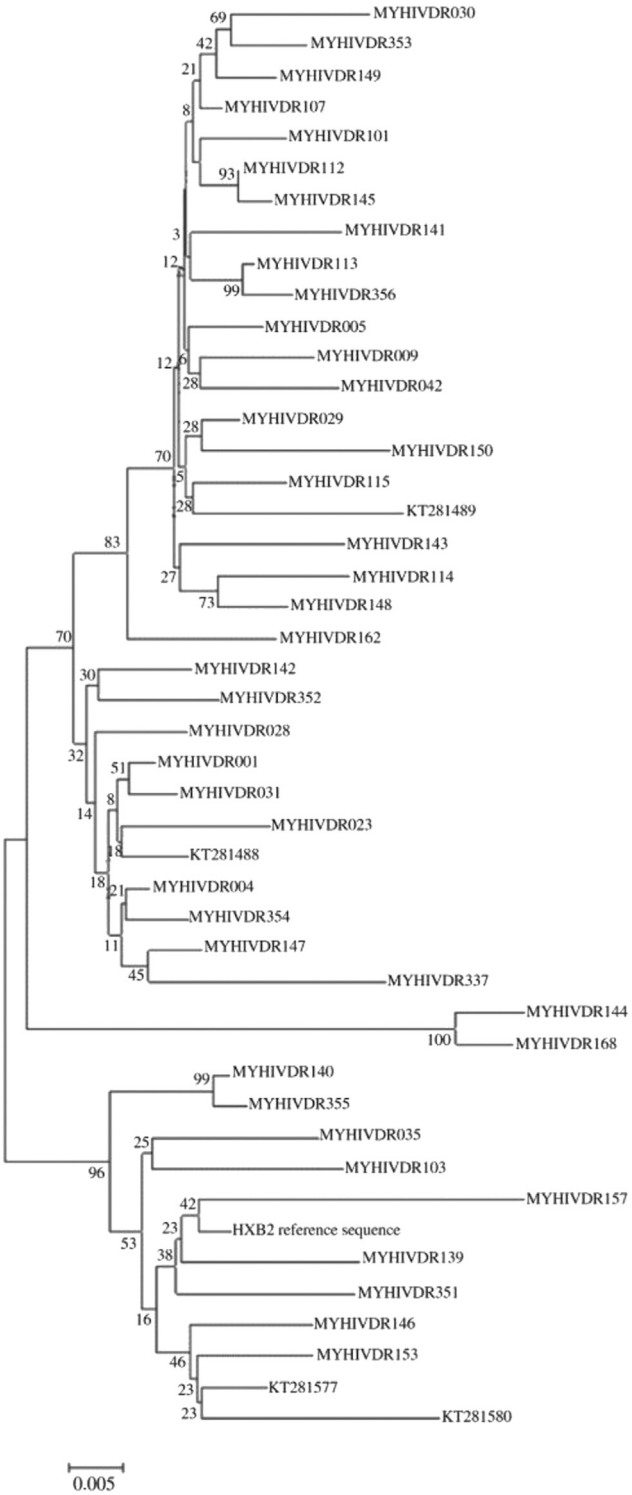

Figure 1. Phylogenetic tree of all 40 samples and the references. The predominant circulating HIV-1 subtype for this population is CRF01_AE.

4. Discussion

This report presents the recent updates on DRMs among naive HIV patients from different parts of Peninsular Malaysia. ART naive patients in this study were defined as patients who were not yet on the ART at the time of sample collection. From this study, the prevalence of TDR against NRTIs, NNRTIs and PI was 7.5% (3/40), 17.5% (7/40) and 17.5% (7/40), respectively. Up till 2012, WHO recommended three categories of transmitted resistance: low (≤5%), moderate (5%–15%) or high (≥15%) [6]. Based on the WHO classification for TDR, Malaysia falls in between moderate to high group. These figures were higher as compared to the previous report which stated that TDR in Asian countries was <5% [4]. These findings were in agreement with the previous report, where DRMs were seen in 14.3% of the 30 prisoners in Kelantan, one of the Eastern states of Peninsular Malaysia [7]. Meanwhile, a comparable finding of TDR among HIV-1 naive patients was also reported by Ong *et al* [8]. However, the report was mainly focused on treatment naive patients in Central part of Peninsular Malaysia, after five years of commencing of HAART treatment in Kuala Lumpur.

Similar findings for PIs resistance were observed in this study and Ong *et al* study [8]. Both studies showed the most two commonest DRMs for PIs were L10I and A71V. The detection of L10I could diminish the PIs susceptibility and might enhance the HIV replication with the existence of other PIs mutations which might further complicate in optimizing treatment options for HIV patients [9]. Currently, the registered PIs in Malaysia are Atazanavir, Ritonavir, Darunavir, Saquinavir and Lopinavir/ritonavir and the current first line ARV recommendation for PI-based regimens for adults as stated in the Consensus Guidelines on Antiretroviral Therapy 2014 for Malaysia is Atazanavir/r plus T DF/FTC and Lopinavir/ritonavir plus T DF/FTCa [10]. Mean while, A71V, a polymorphism that can be detected in a small percentage of treatment naive patients [9], was detected in HIV-1 subtype B.

The study also showed that only 70% of patients were found to have a baseline HIV viral load value prior to ARV treatment. In Malaysia, this HIV viral load is not a routine test prior to commencement of ARV as it is considered as an expensive test. At present, current recommendations for performing HIV viral load are once in 4–6 months after initiation of ART in order to assess treatment response and for early detection of treatment failure, in 6–12 months in patients who have achieved virological suppression for than one year and before the treatment regime is changed [10]. A thorough history is mandatory for all patients with HIV infection especially on the pre and post-exposure prophylaxis as it would significantly assist in the interpretation of the HIV DRMs data.

Currently, the test is mainly focused on HIV-1 patients with evidences of virological failure, with HIV-1 viral load more or equal to 1 000 copies/mL and only available at our laboratory. Mean while, the main issue in executing this test in the newly diagnosed HIV-1 patients is the resources. However, targeting the treatment naive HIV-1 patients should be the utmost plan for the near future and should be considered at the soonest possible time as transmitted drug resistance among HIV-1 naive patients imposes an enormous risk of ARV drug resistance if it not vigilantly monitored. Clearly, these findings were based on a very small sample size and the distribution of samples from each region was not equal. Hence, certainly more samples and data from the various regions in Malaysia need to be accumulated and analyzed.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Sha'ari N. *Summary on update of responses to HIV & AIDS*. Malaysia: Ministry of Health Malaysia; 2015.
[2] World Health Organisation WHO/HIV/2016.02. Global action plan for HIV drug resistance 2016–2021. Geneva: WHO; April 2016. Available from: http://www.who.int/hiv/pub/drugresistance/hiv-drug-resistance-brief-2016/en/.
[3] UNAIDS. 90-90-90 An ambitious treatment target to help end the AIDS epidemic. JC2684. Geneva, Switzerland: UNAIDS; 2014. Available from: http://www.unaids.org/sites/default/files/media_asset/90-90-90_en_0.pdf.
[4] Trotter AB, Hong SY, Srikantiah P, Abeyewickreme I, Bertagnolio S. Systematic review of HIV drug resistance in the World Health Organization Southeast Asia region. *AIDS Rev* 2013; **15**(3): 162.
[5] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725-2729.
[6] Bennett DE, Myatt M, Bertagnolio S, Sutherland D, Gilks CF. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. *Antivir Ther* 2008; **13**(Suppl 2): 25-36.
[7] Ariffin TAATM, Mohamad S, Yusuf WNW, Shueb RH. Antiretroviral drug resistance and HIV-1 subtypes among treatment-naive prisoners in Kelantan, Malaysia. *J Infect Dev Ctries* 2014; **8**(8): 1063-1067.
[8] Ong LY, Razak SN, Lee YM, Sri La Sri Ponnampalavanar S, Syed Omar SF, Azwa RI, et al. Molecular diversity of HIV-1 and surveillance of transmitted drug resistance variants among treatment Naïve patients, 5 years after active introduction of HAART in Kuala Lumpur, Malaysia. *J Med Virol* 2014; **86**(1): 38-44.
[9] HIV drug resistance database. Drug resistance summaries. Stanford: Stanford University Medical Center. Available from: http://hivdb.stanford.edu/.
[10] Consensus guidelines on antiretroviral therapy. Sungai Panjang: The Malaysian Society for HIV Medicine; 2014. Available from: http://www.hivpolicywatch.org/duremaps/data/guidelines/MalaysiaARTguidelines2014.pdf.
[11] Sohn AH, Srikantiah P, Sungkanuparph S, Zhang F. Transmitted HIV drug resistance in Asia. *Curr Opin HIV AIDS* 2013; **8**(1): 27-33.
[12] Kotaki T, Khairunisa SQ, Witaningrum AM, Sukartiningrum SD, Diansyah MN, Rahayu RP, et al. HIV-1 transmitted drug resistance mutations among antiretroviral therapy-Naïve individuals in Surabaya, Indonesia. *AIDS Res Ther* 2015; **12**(1): 1-7.