



The Brazilian Journal of INFECTIOUS DISEASES

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Brief Communication

Assessing subtypes and drug resistance mutations among HIV-1 infected children who failed antiretroviral therapy in Kelantan, Malaysia

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ARTICLE INFO

Article history:

Received 15 January 2012

Accepted 26 February 2012

Keywords:

HIV-1 subtype

Resistance mutation

Children

A B S T R A C T

Antiretroviral (ARV) therapy has dramatically reduced morbidity and mortality in human immunodeficiency virus 1 (HIV-1) infected children. However, development of ARV resistance in these children is a major public health problem due to lack of availability of and access to new drugs. This study was conducted in order to identify circulating HIV subtypes and recombinant forms and evaluate the drug resistance mutation patterns in 18 HIV-1 infected children failing ARV treatment in Kelantan, Malaysia. Genotyping for codon 1-99 of protease (PR) and 1-250 of reverse transcriptase (RT) were performed by polymerase chain reaction (PCR) amplification and DNA sequencing. Subsequently, these were phylogenetically analyzed to determine the subtypes. CRF33_01B (44.4%) was found to be the predominant HIV subtype, followed by B (27.8%), CRF15_01B (16.7%) and CRF01_AE (11.1%) subtypes. The most prevalent RT mutations were T215F/V/Y (66.7%), D67G/N (55.6%), K219Q/E/R (44.4%), M184V/I (38.9%), K70R/E (27.8%) and M41L (27.8%), associated with nucleoside reverse transcriptase inhibitors (NRTI) resistance; and K103N (55.6%), G190A (33.3%), and K101P/E/H (27.8%) associated with non-nucleoside reverse transcriptase inhibitors (NNRTI) resistance. The results showed a possible emergence of CRF33_01B as current predominant subtypes/circulating recombinant forms (CRFs), and a high frequency of primary mutations among HIV-1 infected children after failure of ARV therapy in Kelantan, Malaysia.

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Introduction

In Southeast Asia, 3.5 million people are living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), mostly in Indonesia, Myanmar, Nepal, and Thailand. Regionally, women constitute 37% of the 3.5 million people living with HIV/AIDS, and about a third of infants born to HIV-positive mothers could acquire HIV. At

the end of 2010, it was estimated that 150,000 children in South and Southeast Asia were living with HIV.¹

Mother-to-child transmission (MTCT) is a significant HIV transmission route to children in Asia. Ninety percent of MTCTs occur during late pregnancy, when maternal blood enters the fetal circulation, or during labor and delivery due to mucosal exposure to the virus. Available drugs used for treatment include nucleoside reverse transcriptase inhibitors

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(NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PI), and fusion inhibitors. Infants and children are more susceptible to virological failure with the subsequent risk of developing drug resistance, one of the major obstacles in antiretroviral (ARV) therapy.²

HIV-1 is divided into four distinct groups: M (major), O (outlier), N (non-M non-O or new) and P. The M group is associated with global pandemic and has various subtypes (A-D, F-H, J) and circulating recombinant forms (CRFs).³ The early phase of HIV-1 epidemic in Malaysia was characterized by the prevalence of subtypes CRF01_AE and B among heterosexuals and injecting drug users (IDU), respectively.^{4,5} However, in recent years, co-circulation and subsequent continual recombination events of CRF01_AE and subtype B' (a subtype B variant of Thai origin) have formed new radiations of novel CRFs, including CRF33_01B⁶ and CRF15_01B.⁷

The state of Kelantan is located on the northeastern part of peninsular Malaysia and shares a border with Thailand on the north. Despite its small population of 1.6 million, Kelantan has among the highest number of newly reported HIV cases and women affected by HIV-AIDS in Malaysia. In many instances, HIV transmission in this area starts with infected men transmitting the virus to their wives, who in turn transmit it to their children.⁸

Previous studies on the prevalence of HIV-1 subtypes and drug resistance associated mutations in Malaysia were conducted mainly in adults. Thus, data on HIV-1 infected Malaysian children is missing, although HIV drug resistance analysis is currently essential to guide ARV therapy strategies. This study aimed at analyzing for the first time the circulating HIV subtypes and recombinant forms and the magnitude of primary mutations associated with ARV drug resistance among children who were suspected to have failed their current ARV regimens in the east coastal area of Malaysia.

Materials and methods

A total of 30 plasma samples were collected from HIV-1 infected children who failed treatment at the pediatric clinic of the Hospital Raja Perempuan Zainab II (HRPZ II), Kelantan, Malaysia, from May 2009 to December 2010. Patients received a combination of at least three ARV agents, including NRTIs and one NNRTI or boosted PI. Data on patients' ARV status such as CD4+ count, viral load, ARV therapy, and others were collected from the patient management database. 5 mL blood samples were collected in ethylenediaminetetraacetic acid (EDTA) blood collection tubes. Plasma was separated by centrifugation at 850 g and stored in multiple aliquots at -80° C until used for ribonucleic acid (RNA) extraction. This study was approved by the Medical Research and Ethical Committee of the Malaysian Ministry of Health (NMRR-08-1390-2886) and by the Research Ethics Committee (Human), Universiti Sains Malaysia – USM/KK/PPP/JEPeM (204.3[1]), and an informed consent was obtained from all patients prior to sample collection.

HIV-1 RNA was extracted from 140 µL of plasma by column purification method (QIAamp Viral RNA Mini Kit, Qiagen – Germany), according to the manufacturer's instructions. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using primers bPOL3 (5'-GTGGAAAGGAAGGACACCAAATGAA-3') and PRRT2 (5'-GCCCAATTCAATTTCCCCACTAAC-3'), which

involved a reverse transcription step at 50°C for 30 min, 95°C for 15 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2.5 min, with a final extension step of 10 min at 72°C. The RT-PCR products were then subjected to nested PCR using primers PRRT3 (5'-ACAAGGAACTGTATCCTTTAGCTT-3') and PRRT4 (5'-TTCTGTATGTCATTGACAGTCC-3') with the following cycling conditions: a denaturation step of 5 min at 94°C, and then 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C, with a final extension at 72°C for 7 min. The polymerase chain reaction (PCR) products were analyzed on 1.5% ethidium bromide stained agarose electrophoresis gel. The resulting PCR-amplified DNA fragments were purified using a PCR Product Purification Kit (Qiagen – Germany). The nucleotide sequences of protease (PR) and reverse transcriptase (RT) were determined by cycle-sequencing dideoxy chain termination method on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems – USA) using two sets of primers, PRRT3/PRRT4 and R580 (5'-CCTTCCTTTCCATTTCTGTACA-3')/E1POL1 (5'-GGACCTACCTGTCAACAT-3').

Sequences were aligned using Clustal W, manually edited with BioEdit version 7.0.0. The subtypes were determined using NCBI Genotyping Tools (reference sequence set 2009).⁹ Resistance-associated mutations were identified using Stanford's HIV drug-resistance database genotypic resistance interpretation algorithms.¹⁰ The association between ARV drug and mutation was analyzed by Fisher's exact test (two-tailed), with a significance level of 0.05 and a confidence level of 95%.

Results

Among the 30 HIV-1 treated children enrolled in this study, the quantitative real-time RT-PCR results showed that only 21 had plasma HIV-1 RNA viral load \geq 1,000 copies/mL. Of those, PR and RT genes were successfully amplified and sequenced in 18 patients. Detailed information on the baseline characteristics of these 18 patients is shown in Table 1. The mean HIV-1 viral load in this group was \log_{10} 4.64 copies/mL with a median of \log_{10} 4.74 copies/mL. The mean CD4+ count was 234 cells/mm³, with a median of 144 cells/mm³. The mean duration of ARV exposure was 30.7 months with a median of 24.0 months. The age range was 3-18 years with nine (50%) males and nine (50%) females. Fourteen (77.8%) and four (22.2%) patients received NRTI plus NNRTI and NRTI plus PI, respectively. Out of 12 unsuccessfully amplified samples, one sample had viral RNA > 1,000copies/mL, three samples harboured viral RNA at < 1000 copies/mL, and eight samples had an unknown viral load.

Recombinant CRF33_01B (44.4%, 8/18) was found to be the predominant HIV subtype, followed by subtypes B in 27.8% (5/18), CRF15_01B in 16.7% (3/18), and CRF01_AE in 11.1% (2/18). Out of four patients who received PR therapy, only one child had resistance-associated major mutation in the PR region (I84V). Analysis of the RT gene revealed various mutations/polymorphisms in the NRTI region in the majority of children at the following positions: T215F/V/Y (66.7%), D67G/N (55.6%), K219Q/E/R (44.4%), M184V/I (38.9%), K70R/E (27.8%), M41L (27.8%), L75L/M (16.7%), L210W (11.1%), L74I (11.1%), and T69N (5.6%). The frequencies of mutations associated with the use of NRTIs are shown in Table 2. The most frequent mutations to

Table 1 – Baseline characteristics of 18 HIV infected children

Characteristics	n (%)	Mean (SD)	Median (IR)
Gender			
Male	9 (50.0)		
Female	9 (50.0)		
Ethnicity			
Malay	18 (100.0)		
Age (months)		9.9 (4.2)	9 (7)
Viral load (copies/mL)			
≤ 10 ⁴	3 (16.7)		
10 ⁴ -10 ⁵	9 (50.0)	82,458 (85,909.0)	54,850 (92,720)
≥ 10 ⁵	6 (33.3)		
CD4+ count (cells/mm ³)			
≤ 50	7 (38.9)		
51-200	3 (16.7)	234 (242.0)	144 (441)
≥ 200	8 (44.4)		
Duration of ARV exposure (months)		30.7 (24.6)	24 (28)
Antiretroviral regimen			
2 NRTI and 1 NNRTI	14 (77.8)		
2 NRTI and 1 PI	4 (22.2)		

HIV, human immunodeficiency virus; SD, standard deviation; ARV, antiretroviral; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors.

Table 2 – Frequency of mutations according to current use of NRTIs and NNRTIs

No. patients/ mutations	NRTI									
	12		12		5		5		1	
	3TC	(%)	AZT	(%)	D4T	(%)	DDI	(%)	TDF	(%)
M41L	1	(8.3)	2	(16.7)	3	(60)	4	(80)	0	(0)
D67DN/G/N	7	(58.3)	7	(58.3)	2	(40)	2	(40)	1	(100)
T69N	1	(8.3)	0	(0)	0	(0)	0	(0)	1	(100)
K70E/R	4	(33.3)	5	(41.7)	0	(0)	1	(20)	0	(0)
L74I	1	(8.3)	1	(8.3)	1	(20)	1	(20)	0	(0)
V75L/M	2	(16.7)	1	(8.3)	2	(40)	1	(20)	0	(0)
M184I/V	7	(58.3)	7	(58.3)	0	(0)	0	(0)	0	(0)
L210W	0	(0)	1	(8.3)	1	(20)	2	(40)	0	(0)
T215CFSY/F/V/Y	7	(58.3)	7	(58.3)	4	(80)	4	(80)	1	(100)
K219E/Q/R	5	(41.7)	5	(41.7)	2	(40)	2	(40)	1	(100)

No. patients/ mutations	NRTI			
	4		11	
	NVP	(%)	EFV	(%)
A98G	1	(25)	0	(0)
K101E/H/P	0	(0)	5	(45.5)
K103N	1	(25)	8	(72.7)
V106M	0	(0)	1	(9.1)
V108I	1	(25)	1	(9.1)
V179D	0	(0)	1	(9.1)
Y188L	0	(0)	2	(18.2)
G190A	2	(50)	2	(18.2)
P225H	0	(0)	2	(18.2)
P227L	1	(25)	0	(0)
M230L	0	(0)	1	(9.1)

AZT, zidovudine; 3TC, lamivudine; TDF, tenofovir; DDI, didanosine; D4T, stavudine; EFV, efavirenz; NVP, nevirapine; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.

NNRTI were substitutions at K103N (55.6%), followed by G190A (33.3%), K101P/E/H (27.8%), V108I (16.7%), P225H (16.7%), V106M (11.1%), Y188L (11.1%), F227L (11.1%), A98G (5.6%), V179D (5.6%), and M230L (5.6%). The frequencies of mutations associated with the use of NNRTIs are shown in Table 2.

Fisher's exact test revealed a significant association between certain mutations and exposure to NRTIs (data not shown). Significant associations were observed between mutation M184V and zidovudine (AZT) resistance ($p = 0.038$); mutation M184V and lamivudine (3TC) resistance ($p = 0.038$); mutation M41L and didanosine (DDI) resistance ($p = 0.008$); and mutation M41L and 3TC resistance ($p = 0.022$). Only one patient (5.6%) had no mutation at all in the PR and RT gene, while 94.4% patients had resistance mutations to both NRTI and NNRTI.

Discussion

Although the rate of HIV infections has declined in Southeast Asia in recent years, HIV/AIDS still poses a serious public health problem, with children being the most vulnerable group.¹ This study provides the first report on circulating HIV subtypes and recombinant forms, and mutations associated with ARV resistances among HIV-1 children who failed treatment in the east coastal area of Malaysia, which is located next to Thailand, the country with the highest incidence of HIV/AIDS in Southeast Asia.

In this study, recombinant CRF33_01B (44.4%) was found to be the predominant HIV subtype, followed by subtypes B in 27.8%, CRF15_01B in 16.7%, and CRF01_AE in 11.1% in children failing treatment in Kelantan, Malaysia. The results support the recent findings on the emergence of new predominant HIV subtypes/CRFs circulating in Malaysia, demonstrating the dynamic and rapid evolution of HIV. In the early HIV pandemic, subtypes B and CRF01_AE were the major subtypes found in Malaysia. An initial report showed that there was an almost equal prevalence of subtype B (50%) and CRF_AE/B recombinant (41.7%) among IDU in Kuala Lumpur, Malaysia.¹¹ However, in the following years, various studies performed on HIV-1 patients in Kuala Lumpur reported a predominance of subtype CRF01_AE (65-86%) over subtype B (8.3-14%).^{6,12-14} In addition, the emergence of second generation derivatives resulting from co-circulation of CRF01_AE and subtype B, CRF33_01B, as well as other URFs, were also reported in these studies, albeit at lower prevalence. Interestingly, the present study, which attempted for the first time to describe the molecular epidemiology of HIV-1 among children in Kelantan, showed that CRF33_01B has emerged as the predominant circulating subtype in the state. The higher prevalence of CRF33_01B and lower prevalence of CRF01_AE demonstrated in this study is in accordance with a recent proposition regarding a possible gradual reduction of the current predominance of CRF01_AE and greater distribution of CRF33_01B in Malaysia.¹⁵

Only one major mutation (I84V) associated with PI was observed in a patient. In contrast, most of these children carried mutations to NRTIs and NNRTIs at higher frequencies. The mutation rate of resistance to both NRTI and NNRTI was 94.4%, with the most frequent mutations including types T215F/Y/V, D67G/N, M184V/I, and K219Q/E/R (NRTI), and K103N (NNRTI). The higher frequencies of resistance mutations observed in

RT than in PR shown in the present study are similar to those previously reported in Kuala Lumpur,⁶ Thailand^{16,17} and India¹⁸ (RT resistant > PR resistant). It is possible that the acquisition of resistance mutations in PR is difficult because most of the sites in PR are under strong selection, making it less susceptible to resistance mutations.¹⁸ Alternatively, the lower frequencies of mutation in PR observed in many studies, including the present study, could be due to a more widespread use of double NRTI with NNRTI rather than with PI as the first line regimen of treatment in patients.

Extensive drug resistance to NRTIs, NNRTIs, and PI in HIV-1-infected children is a global phenomena observed in various studies, leaving these children with limited choice of ARV drugs for their second line or salvage therapy.^{19,20} Almeida et al. observed HIV resistance to NRTIs, NNRTIs, and PIs in 96%, 61%, and 17% of 23 ARV-experienced children in São Paulo, Brazil, respectively.²⁰ The RT mutation K103N was the most commonly occurring NNRTI-resistance mutation observed in the current study. K103N is associated with resistance to efavirenz, the main NNRTI used in these children. An almost universal presence of NRTI resistance mutations, particularly T215Y/F, M184V/I, D67N, M41L, and K219Q/E was detected among children receiving ARV therapy in São Paulo, Brazil, a finding similar to this study's.²⁰ Interestingly, contrary to a report on the presence of NRTI resistance mutation, Q151M, in 5% of HIV-1 infected Thailand children,¹⁶ this clinically important mutation was not found in the present study. A Q151M multi-drug resistance complex is composed of the Q151M mutation, which is normally the first to appear, followed by at least two of the following four mutations: A62V, V75I, F77L, and F116Y. The Q151M MDR confers resistance to almost all NRTIs with the exception of tenofovir, rendering NRTI therapy ineffective in patients with this mutation.¹⁹

In conclusion, the emergence of CRF33_01B as the predominant HIV-1 subtype among children who failed ARV therapy in Kelantan, Malaysia, is reported in this study, an observation not previously described. Furthermore, a high frequency of major mutations associated with resistance to ARV drugs in these children was described. Although the sample size analyzed in this study was rather small, reflecting the smaller number of HIV-1 infections in children and perhaps warranting further investigation, the results are still invaluable as they provide pertinent information on the presence of various HIV-1 subtypes and the status of HIV-1 drug-resistance in this area. It remains to be investigated whether or not similar HIV-1 subtypes and ARV drug resistance patterns would be observed in adults in Kelantan.

Sequence data

The nucleotide sequences reported in this paper have been submitted to the GenBank database (accession numbers JN596254-JN596271).

Acknowledgements

The authors thank Prof. Dominic Dwyer and Dr. Choo Beng Chew from the Centre for Infectious Diseases and Microbiology Laboratory Services, Westmead Hospital, Australia for their

technical assistance. The authors are deeply indebted to the patients who agreed to participate in this study and to the staff nurse, Ramlah Abdul Rahman at the Pediatric Clinic, Hospital Raja Perempuan Zainab II, Kota Bharu, Kelantan, Malaysia, for her assistance in this study. The study was supported by a Research University Grant (Grant number: 1001/PPSP/812029).

Conflict of interest

All authors declare to have no conflict of interest.

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