# HIV-1 DRUG RESISTANCE MUTATIONS IN ANTIRETROVIRAL TREATED THAI PATIENTS WITH LOW VIRAL LOAD

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**Abstract.** Low-level viremia [LLV; HIV-1 viral load (VL) <2,000 copies/ml] in Thailand has risen due to the success of a program of free combined antiretroviral treatment (cART) under the country's Universal Health Coverage Scheme. However, this program resulted in increasing HIV drug resistance mutations (DRMs) in cART failure cases, but there are few or no data available of HIV DRMs in LLV from Thailand. Using a modified commercial drug resistance genotype test (DRGT) to increase sensitivity of DRM detection, the success rate of HIV *pol* amplification and the frequency of HIV DRMs in randomly collected plasma samples ($n$ = 30) was 80% and 47%, respectively. These plasma samples were divided into two equal groups, one group with HIV VL <1,000 copies/ml and the other with VL of 1,000-2,000 copies/ml. HIV-1 *pol* region could be amplified from all (100%) group 1 and 9/15 (60%) group 2 samples. HIV DRMSs were detected in these *pol* sequences from group 1 and group 2 samples at 60% and 56%, respectively. Presence of HIV DRMs against NNRTIs, NRTIs and PIs among the patients was 54%, 37% and 9%, respectively. The most common (37%) HIV DRM against NRTIs was M184V/I. The procedure can be applied in routine screening of HIV DRMs in antiretroviral drug treatment failure patients with low VL. Such data should help to improve and develop clinical management programs towards achieving Thailand National AIDS Strategy to eliminate HIV/AIDS by 2030.

**Keywords:** antiretroviral drug resistance, genotyping, HIV-1, low viral load, LLV

## INTRODUCTION

Widespread use of antiretroviral drug therapy as a part of universal health insurance scheme to end AIDS epidemic in Thailand has depended on combination antiretroviral therapy (cART) to reduce morbidity and mortality of HIV-1 infected patients by suppresses viral load and progression to acquire immunodeficiency syndrome (AIDS) (Jenwitheesuk *et al*, 2003). However, cART can result in a significant increase of antiretroviral drug resistance in HIV-infected patients, which leads to treatment failure (Praparattanapan *et al*, 2011). ART not sufficient

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to completely suppress viral replication imposes selective pressure for emergence of drug-resistant virus mutants, a major problem in HIV-1 treatment (Gallant, 2005; Richman, 2014). HIV drug resistance testing has now become a part of standard management in treating HIV-1 infected patients (Vandamme *et al*, 2011; Gunthard *et al*, 2019).

HIV drug resistance mutations (DRMs) can emerge early after treatment failure, even with low levels of HIV viral load (VL) (Taiwo *et al*, 2011; Delaugerre *et al*, 2012; Thompson *et al*, 2012). The goal of cART is to suppress viral replication to below detection limit of a VL assay, which generally is defined as HIV RNA <20-75 copies/ml (Palella *et al*, 1998; Jordan *et al*, 2013). However, therapy failures are observed in clinical practice characterized by low-level viremia (LLV) with VL <2,000 copies/ml, which constitutes 4-8% of cART-treated patients (Santoro *et al*, 2014). Persistence of LLV lasting at least 6 months doubles the risk of subsequent virologic failure and accumulation of HIV DRMs, which can lead to disease progression (Sklar *et al*, 2002; Gonzalez-Serna *et al*, 2014; Swenson *et al*, 2014). Persistent LLV patients can be associated with increase of immune activation and risk of treatment failure (Karlsson *et al*, 2004; Gonzalez-Serna *et al*, 2014). Thus, the ability to identify HIV DRMs in LLV patients allows a timely change in therapeutic management (Mackie *et al*, 2010).

HIV drug resistance genotype determination is commercially available, *eg* TruGene HIV-1 Genotyping Assay (Siemens Healthcare Diagnostics, Eschborn, Germany) and ViroSeq HIV-1 Genotyping System (Abbott, Wiesbaden, Germany). These assays detect drug-resistance mutations using nucleotide sequences of regions of HIV-1 *pol* [*reverse transcriptase* (RT), *protease* (PR) and *integrase* (IN) regions] gene from plasma samples. Guidelines from most clinical laboratories including US FDA advise testing of HIV drug resistance genes only on specimens with VL ≥2,000 copies/ml using ViroSeq HIV-1 Genotyping System (Abbott) and ≥1,000 copies/ml with TruGene HIV-1 Genotyping Assay (Siemens Healthcare Diagnostics) (Nettles *et al*, 2004; Thompson *et al*, 2012; Richman, 2014). However, HIV DRMs in LLV are difficult to determine because of limitation in HIV *pol* amplification. A number of modified HIV drug resistance genotyping tests (DRGT) to detect DRMs in LLV plasma samples have been developed by modifying RNA concentration coupled with *pol* amplification and sequencing using next generation sequencing techniques to increase *pol* nucleotide sequence yields (Karlsson *et al*, 2004; Waters *et al*, 2006; Mackie *et al*, 2010; Taiwo *et al*, 2011; Assoumou *et al*, 2013; Swenson *et al*, 2014; Santoro *et al*, 2014).

Employing ultracentrifugation to increase LLV plasma viral RNA yield, HIV *pol* fragment was amplified and sequenced using ViroSeq HIV-1 Genotyping System (Abbott). This will enable determination of HIV DRMs prevalence in cART-treated HIV-1- infected patients with LLV for the first in Thailand.

## MATERIALS AND METHODS

### Plasma samples

Plasma samples ($n$ = 30) with HIV RNA VL ≤2,000 copies/ml were randomly collected from all ART HIV-1-infected patients submitted for drug resistance testing to the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand during 2014-2015. HIV-1 viral load was evaluated using Abbott *m*2000 Real Time HIV-1 assay (Applied

Biosystems, Foster city, CA). Samples were divided into two groups based on HIV viral load, namely, group 1 (samples U01-U15) with HIV VL 1,000-2,000 copies/ml and group 2 (H01-H15) with HIV VL <1,000 copies/ml. Plasma samples were kept at -80°C until used.

Study protocols were approved by Institutional Review Board of Siriraj Hospital, Mahidol University (ethics certificate of approval no. si128/2016).

**HIV-1 *pol* (RT and PR amplification, sequencing and analysis)**

HIV-1-positive plasma sample containing VL of $2.76x10^5$ copies/ml quantified using Abbott *m*2000 Real Time HIV-1 (Applied Biosystems) was serial diluted with HIV-1-negative human plasma to $1x10^4$, $5x10^3$, $2.5x10^3$, $1.25x10^3$ and $6.25x10^2$ copies/ml as control. Two ml aliquot of plasma sample was centrifuged at 35,000 *g* at 4°C for 2 hours (Optima L-100XP ultracentrifuge; Beckman Coulter, Brea, CA) (Santoro *et al*, 2014). HIV-1 RNA was extracted from pellet using a guanidine lysis buffer followed by isopropanol/ethanol wash according to ViroSeq RNA extraction protocol (Applied Biosystems). In brief, 300 $\mu$l aliquot of viral lysis buffer (ViroSeq HIV-1 Genotyping System; Abbott) was added to each viral pellet, vortexed for 10 seconds, incubated at 25°C for 10 minutes. Then lysate was added to an equal volume of isopropanol, vortexed for 10 seconds and centrifuged at 13,000*g* for 20 minutes at room temperature, added with 500 $\mu$l of cold 70% ethanol, and centrifuged at 13,000*g* for 5 minutes at room temperature. Virus pellet was air-dried, resuspended in cold 50 $\mu$l of cold RNA diluent (ViroSeq HIV-1 Genotyping System; Abbott) and stored at -80°C until used.

Partial HIV-1 *pol*, 1,800 bp [encoding RT codons 1-335 and protease (PR) codons 1-99) sequences], was amplified by RT-PCR and sequenced using ViroSeq HIV-1 Genotyping System (Applied Biosystems). HIV-1 RT and PR nucleotide sequences were aligned with HIV CRF01_AE CM240 reference strain (GenBank no. U54771.1) employing a ViroSeq version 2.0 software (Applied Biosystems). Sequences were deposited at GenBank, accession nos. MN402834-MN40285. Mutations in HIV-1 RT and PR sequences associated with HIV drug resistance genotypes were identified using a HIV drug resistance database (http://hivdb.stanford.edu/).

**Statistical analysis**

Mann-Whitney *U* test was used for comparing numbers of HIV drug resistance mutations reported between group 1 and group 2 samples. Statistical analysis was carried out employing standard functions of Statistics Package for the Social Sciences (SPSS) version 25 (IBM, Armonk, NY).

## RESULTS

**HIV-1 yield**

Ultracentrifugation sedimentation of HIV-1 virus particles from (2 ml) plasma samples yielded among group 1 samples a range from 1,069 (U14) to 1,968 (U09) copies/ml and from 370 (H07) to 972 (H10) copies/ml among group 2 (Table 1).

**HIV-1 drug resistance mutations**

Success rate of HIV-1 *pol* (PR and RT regions) amplification was 24/30 (80%), 100% and 60% for group 1 and 2 samples, respectively (Table 1). Comparison of the PR and (partial) RT nucleotide sequences with those deposited in HIV Drug Resistance Database (http://hivdb.stanford.edu/) revealed a frequency of HIV DRMs in LLV plasma samples of 60% (9/15) and 56% (5/9) in group 1 and group 2, respec-

Table 1
HIV drug resistance mutations among low-level viremia HIV-1 infected Thai patients.

| Sample ID | CD4+ count (cells/mm$^3$) | Viral load (copies/ml) | ART | HIV-1 *pol* sequenced[a] | HIVDR mutation in ART | | |
|---|---|---|---|---|---|---|---|
| | | | | | PI[b] | NRTI[c] | NNRTI[c] |
| Group 1 (viral load 1,000-2,000 copies/ml) | | | | | | | |
| U01 | 215 | 1,797 | AZT, NVP, 3TC | + | - | - | Y188L |
| U02 | 79 | 1,577 | AZT, NVP, 3TC | + | - | - | Y188L |
| U03 | 136 | 1,610 | d4T, EFV, 3TC | + | - | - | K103N, Y188L |
| U04 | 423 | 1,388 | AZT, LPV/RTV, TDF | + | - | - | K103N, P225H |
| U05 | 566 | 1,285 | RPV, TDF, 3TC | + | - | - | - |
| U06 | 382 | 1,479 | AZT, LPV/RTV, 3TC | + | - | - | - |
| U07 | 363 | 1,537 | LPV/RTV, TDF, 3TC | + | - | K65R, L74V, M184V | G190V |
| U08 | 302 | 1,206 | AZT, LPV/RTV, RPV | + | I54V, L76V, G73S, L89V | D67N | - |
| U09 | 155 | 1,968 | AZT, LPV/RTV, 3TC | + | - | D67G, M184V | V108I, Y181C, H221Y |
| U10 | 199 | 1,268 | NVP, TDF, 3TC | + | - | - | - |
| U11 | 70 | 1,148 | AZT, LPV/RTV, TDF | + | - | - | - |
| U12 | 136 | 1,072 | LPV/RTV, TDF, 3TC | + | - | - | - |
| U13 | 334 | 1,174 | NVP, TDF, 3TC | + | - | K65R, L74I, Y115F, M184V | V108I, Y181C, H221Y, Y318F |
| U14 | 288 | 1,069 | LPV/RTV, TDF, 3TC | + | - | - | K103N, V106L, |
| U15 | 316 | 1,860 | AZT, LVP/RTV, TDF | + | - | - | - |
| Group 2 (viral load <1,000 copies/ml) | | | | | | | |
| H01 | 246 | 841 | AZT, LPV/RTV, TDF | + | - | - | - |
| H02 | 236 | 857 | LPV/RTV, TDF, 3TC | + | - | M184V | Y188L |
| H03 | 321 | 940 | AZT, LPV/RTV, RPV | + | L33F | M184I | K103N, V179D, Y181C, G190A, H221Y |

Table 1 (Continued)

| Sample ID | CD4+ count (cells/mm3) | Viral load (copies/ml) | ART | HIV-1 *pol* sequenced[a] | HIVDR mutation in ART | | |
|---|---|---|---|---|---|---|---|
| | | | | | PI[b] | NRTI[c] | NNRTI[c] |
| H04 | 169 | 685 | AZT, NVP, 3TC | - | - | - | - |
| H05 | 420 | 567 | AZT, NVP, 3TC | - | - | - | - |
| H06 | 336 | 610 | NVP, TDF, 3TC | + | - | K65R, M184V, T215S | Y188L |
| H07 | 156 | 370 | AZT, LPV/RTV, RPV | + | - | - | - |
| H08 | 340 | 649 | AZT, LPV/RTV, TDF | + | - | - | - |
| H09 | 250 | 713 | AZT, LPV/RTV, TDF | + | - | K65R, T215I | K103N, G190A |
| H10 | 167 | 972 | AZT, LPV/RTV, 3TC | + | - | - | - |
| H11 | 348 | 640 | AZT, LPV/RTV, TDF | + | - | D67N, M184V | V108I, Y181C, H221Y |
| H12 | 273 | 619 | d4T, EFV, 3TC | - | - | - | - |
| H13 | 332 | 962 | AZT, NVP, 3TC | - | - | - | - |
| H14 | 453 | 562 | AZT, NVP, 3TC | - | - | - | - |
| H15 | 265 | 649 | EFV, FTC, TDF | - | - | - | - |

[a]Complete *PR* and partial *RT* sequences. [b]In *PR* sequence. [c]In partial *RT* sequence.

ART, antiretroviral drug treatment; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; FTC, emtricitabine; LPV/RTV, lopinavir/ritonavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PI, protease inhibitor; RPV, rilpivirine; TDF, tenofovir; 3TC, lamivudine.

tively and the prevalence of HIV DRMs in all thirty samples was 40% (Table 1).

Frequency of HIV DRMs against nucleoside reverse transcriptase (NRTIs), non-nucleoside reverse transcriptase (NNRTIs) and protease (PIs) inhibitors among 52 DRMs was 37% ($n$ = 19), 54% ($n$ = 28) and 9% ($n$ = 5), respectively (Table 1). Most frequent HIV DRMs against NRTIs were M184V/I (37%) from nineteen lamivudine (3TC)-treated patients, with an emergence rate of 37% (7/19). Frequency of K103N/S, Y181C, Y188L, H221Y NNRTIs mutation was 18% ($n$ = 5), 14% ($n$ = 4), 18% ($n$ = 5) and 14% ($n$ = 4), respectively. Resistance mutations I54V, L76V, G73S, L89V and L33F in PR were present in only two (22%) from nine cARTs with protease inhibitors treated LLV plasma samples.

## DISCUSSION

Inclusion of an ultracentrifugation concentration step of HIV-1 particles from LLV plasma prior to application of

a commercial HIV-1 genotyping system (ViroSeq, Abbott) resulted in 100% genotyping success rate with plasma samples containing HIV VL of 1,000-2,000 copies/ml and 60% in samples with VL of <1,000 copies/ml. These results are comparable with other studies on samples with low viremia (<2,000 copies/ml). The success rate of HIV-1 genotyping detection in LVL plasma samples varies from 33.0-97.3% depending on HIV viral load and methods used for RNA extraction, HIV-1 *pol* amplification and sequencing (Sturmer *et al*, 2003; Steegen *et al*, 2006; Costagliola *et al*, 2007; Taiwo *et al*, 2011; Delaugerre *et al*, 2012; Gonzalez-Serna *et al*, 2014; Santoro *et al*, 2014). Comparing the same extraction and amplification techniques, HIV-1 *pol* positive rate in HIV-1 VL<1,000 copies/ml samples is lower than those samples with VL>1,000 copies/ml, *eg* 76% *vs* 91.8% with High Pure nucleic acid extraction kit (Roche Molecular Biochemicals, Mannheim, Germany) and in-house RT-PCR (Steegen *et al*, 2006), 82.8% *vs* 97.3% with modified ViroSeq system (Santoro *et al*, 2014), 87.7% *vs* 100% with NucliSENS easyMAG nucleic acid extraction kit (bioMerieux) and in-house nested RT-PCR (Gonzalez-Serna *et al*, 2014).

The frequency of HIV DRMs in PR and (partial) RT sequences detected in LLV plasma samples was lower than that reported from Canada (88% using next generation sequencing), France [90% for LLV >1,000 copies/ml and 70% for LLV <1,000 copies/ml using TruGene system (Siemens Healthcare Diagnostics) and Italy (96.4% using modified Viroseq system; Abbott), but lower than from USA (30% using Sanger sequencing) (Gonzalez-Serna *et al*, 2014; Swenson *et al*, 2014; Assoumou *et al*, 2017). The most frequent DRMs (M184I/V) against NRTIs in LLV subjects were similar to that (53.1%) detected in a surveillance of 24,279 blood plasma samples collected from 1999 to 2014 in the country, followed by DRMs associated NNRTIs Y181I/C (31.4%) mutations (Iemwimangsa *et al*, 2017), while we found the next highest frequent mutations to be K103N/S and Y188L. In LLV with long-term NNRTIs treatment, K103N/S DRMs virus might survive longer than any other DRMS associated NNRTIs viruses due to better viral fitness and replication (Wang *et al*, 2010; Xu *et al*, 2010). Of the three NNRTIs associated DRMs, namely, K103N, Y181C and G190A, K103N resistance mutations can inhibit available NNRTIs better than any other DRMs, up to 20-50 folds, and effectively replicate similar to that of wild type HIV-1 *in vitro* (Joly *et al*, 2000; Wang *et al*, 2010; Xu *et al*, 2010).

The 90-90-90 strategy launched by UNAIDS is to attain 90% of all people living with HIV to become aware of their infection status by 2020, 90% of all people with diagnosed HIV infection to receive sustained antiretroviral therapy by 2020 and 90% of all people receiving antiretroviral therapy to achieve viral suppression by 2020 (UNAIDS, 2017). To this end, Thailand has launched a 2017 - 2030 National AIDS Strategy to end AIDS epidemic in the country using the 90-90-90 strategy by 2030 (UNAIDS, 2017). There are 450,000 individuals in Thailand with HIV infection in 2017, 72% of whom are on cART, with 86% of treated patients demonstrating viral load suppression (UNAIDS, 2018). However, as low viral load increases there will be an accumulation of drug resistance mutations in these cART populations, jeopardizing success of this National AIDS strategy.

This study shows an ultracentrifugation step that can be performed in most clinical laboratories to enhance viral yield

allowed 90% success rate in genotyping HIV-1 *pol* to detect antiretroviral resistance mutations in plasma of low-level viremia patients, which was not a rare occurrence. The simple improvement in detecting drug resistance HIV-1 genes in treated patients with low viral load should be included in the national strategy to assist in realizing Thailand 90-90-90 Strategy to eliminate HIV/AIDS by 2030.

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