SURVEILLANCE OF HIV-1 DRUG-RESISTANCE MUTATIONS IN THAILAND FROM 1999 TO 2014

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Abstract. Antiretroviral resistance has long been a serious problem in Thailand. In order to monitor developmental rate of mutations and its impact of the national policy, frequency of drug-resistance mutations in HIV-1 reverse transcriptase (RT) and protease (PR) were analyzed from 24,279 blood plasma samples collected from 1999 to 2014. HIV-1 drug resistance mutations were influenced by drugs that have been used widely as first-line regimens. M184I/V was the most common (53.1% prevalence) RT inhibitor (NRTI) mutation. Other NRTI-associated mutations increased dramatically after the Universal Coverage Scheme was launched in 2007, but declined on the whole after introduction of the Thai National Guidelines in 2010. However, non-NRTI-associated mutations increased between 1999 and 2007, but have remained constant since, with Y181I/C the most (31.4%) prevalent. PR drug-associated mutations (M36I/L/V, H69K/R and L89I/M/V) previously considered as CRF01_AE polymorphisms constituted > 90% prevalence in all samples. The launch of antiretroviral treatment influenced the pattern of mutations and the Universal Coverage Scheme also impacted the rate of development of resistance mutations on a national scale. Drug resistance trends in Thailand could be ascribed to drug regimens that have been used for over a decade. Results from this study can be used as indicators of the success of the Universal Coverage Scheme. Knowledge of the trend of HIV drug-resistance mutations, past and present, is essential in formulating an effective antiretroviral treatment strategy.

Keywords: HIV-1 mutation, Universal Coverage Scheme, Thailand

INTRODUCTION

Highly active anti-retroviral therapy (HAART) has become the main means in treating and preventing viral transmission for HIV-1 infected patients in Thailand (Sutthent *et al*, 2005). The Thai Ministry of Public Health launched the National Access to Antiretroviral Program for People Living with HIV/AIDS (NAPHA) in 2002, with the aim of providing proper and effective antiretroviral therapy to all Thai patients with HIV infection (Chasombat *et al*, 2009). In 2006, the Universal Coverage (UC) scheme, conducted by the National Health Security Office (NHSO), also launched a provision of preventive

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care for all Thai citizens, with focus on health promotion and disease prevention at extremely low healthcare cost (deHaan, 2007). Coverage to include antiretroviral (ARV) treatment for HIV/AIDS has been extended since 2007, when the National AIDS Program (NAP) fully covered all necessary HIV/AIDS treatment and care (Hirsch *et al*, 1998; National Health Security Office, 2008; Chasombat *et al*, 2009; Center for Health Market Innovations, 2015).

Medical services for HIV/AIDS include antiretroviral therapy, treatment of hyperlipidemia, laboratory service (such as viral load test, blood chemistry analysis and HIV drug resistance test), counseling service, and promotion of condom use (National Health Security Office, 2008, 2011). According to current guidelines, CD4 counts can be performed twice yearly together with plasma viral load test without charge: twice in the following first year of detecting HIV infection, and once yearly thereafter. Patients who have a history of anti-HIV medication and adhere to ARV protocol correctly and consistently and still have viral load > 2,000 copies/ml (according to the 2010 Thai National Guideline recommendation) are eligible for HIV drug resistance testing once yearly free of charge (Sungkanuparph and Techasathit, 2011). However, HIV drug treatment has resulted in the emergence of drug-resistant strains (Sutthent *et al*, 2005). After initiating NAP, HIV-1-resistance mutations to HAART have appeared, reducing efficacy of AVR treatment, and has become a public health problem (Weinstein *et al*, 2001; Sax *et al*, 2005; Booth and Geretti, 2007; Sukasem *et al*, 2007; Yazdanpanah *et al*, 2007).

In order to determine the trend of HIV-1 drug-resistant mutations, this study monitored frequency of HIV-drug resistance by performing a large scale retrospective study of Thai HIV-infected patients covering a period of over a decade. HIV genotyping was performed on all samples from 1999 to 2014.

MATERIALS AND METHODS

Study samples

A total of 24,279 EDTA blood samples collected from 1999 to the end of 2014 from large public medical centers, representing different geographical areas of Thailand were obtained from the Virology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. This study was conducted with approval of the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (MURA2014/389).

Genotypic drug-resistance assay

Genotypic drug-resistance assay was carried out on blood plasma samples with a HIV viral load > 2,000 copies/ml (Sungkanuparph and Techasathit, 2011). HIV-1 RNA was sequenced using TRUGENE® HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, Tarrytown, NY). HIV protease (PR) and reverse transcriptase (RT) gene sequences were analyzed and interpreted for drug resistance mutations using Stanford HIV Drug Resistance Database and referring to list of drug-resistant mutations recommended by the International AIDS Society-USA (Wensing *et al*, 2014). HIV-1 subtypes were identified using Stanford Interpretation System (http://hivdb.stanford.edu).

Statistical analysis

Prevalence of HIV drug resistance within a calendar year is reported as percentage with 95% CI. Presence of drug resistance with respect to the Thai govern-

ment policy was tested using chi-square test for trend compared to frequency of resistance across a given period of time. An α value of 0.05 was used to determine statistical significance. Data were analyzed using Epitools epidemiological calculator (http://epitools.ausvet.com.au/).

RESULTS

Study population

This large scale retrospective study examined plasma samples from chronically drug-treated HIV-1 individuals, who had undergone at least one genotypic antiretroviral drug resistance test per calendar year from 1999 to 2014. Tests for RT and PR gene mutations were carried out on 24,245 (99.86%) and 22,976 (94.63%) samples, respectively. The major (91.18%) subtype was CRF01_AE, which is predominant in Southeast Asia, and 7.06% of subtype B. Other subtypes were A (0.37%), C (0.19%), D (0.21%), G (0.03%), and K (0.07%). CRF02_AG subtype constituted 0.20%.

HIV-1 drug-resistant mutations from 1999 to 2014

In accordance with the recommended list of the International AIDS Society-USA (Wensing *et al*, 2014), the total frequency of HIV-1 resistance-associated mutations is presented separately with drug class (Fig 1). Throughout 16 years of observation, thymidine analogue mutations (TAMs) were common findings. As a group of mutations, TAMs caused multiple resistance to RT inhibitors (NRTIs), being present in > 10% of all samples [M41L (11.5%), D67N (14.7%), K70R (12%), T215FY (15.9%), and K219E/Q (10.8%)]. K65R/E/N mutation was in 5.6% of the samples and the remaining had < 5% prevalence (Fig 1a). M184I/V was the most prevalent (53.1%) RT mutation associated closely with resistance to lamivudine, a widely used ARV in Thailand (Cohen, 2003; Puthanakit *et al*, 2005).

Y181I/C/V was the most common (31.4%) RT mutation against nevirapine (NVP), efavirenz (EFV), etravirine (ETR), and rilpivirine (RPV) followed by K103N/S (23.8%), G190A/S (20.9%) and K101E/H/P (13.6%). However, other NNRTI mutations (V108I, H221Y, A98G, V179D/F/L/T, V90I, Y188C/H/L, P225H, M230I/L, E138A/G/K/R, V106A/I/M, L100I, and F227C) were present at < 10% (Fig 1b).

Resistance mutations in PR showed an extremely high prevalence of M36I/L/V, H69K/R, and L89I/M/V mutations. Each of these mutation were present > 90% in total samples (95.4%, 92.7%, and 93.2%, respectively) (Fig 1c). These mutations lie within the PR active site (Wensing *et al*, 2014). L10C/F/I/R/V, K20I/M/R/T/V, G16E, L63P, I93L/M, V82A/F/I/L/S/T, I62V, V77I, D60E, I64LM/V, M46I/L, L33F, A71I/L/T/V, and I54A/L/M/S/T/V mutation was found in 29.3%, 28.3%, 25.2%, 23.0%, 15.3%, 10.8%, 8.9%, 3.6%, 3.2%, 3.0%, 2.9%, 2.6%, 2.6%, and 1.7% of samples, respectively. The other PR mutations (L90M, I84V, V11I, Q58E, L76V, I47A/V, F53L, N83D, G48V, L24I, T74P, N88D/S, G73A/C/S/T, K43T, V32I, I50L/V, I85V, E34Q and D30N) were present at < 1%.

Changes in HIV-1 drug-resistant mutations during 1999-2014

Overall, the prevalence of NRTI individual drug resistant mutations has decreased significantly over the period of the survey (Fig 2a). TAMs, M41L, D67N, K70R, L210W, T215Y/F and K219E/Q, was prevalent at 20-50% between 1999 and 2003 and remained < 30% after 2007 before decreasing to < 10% at the end of 2014. However, mutation M184I/V, the most prevalent (52.9%) in 1999, before reduc-

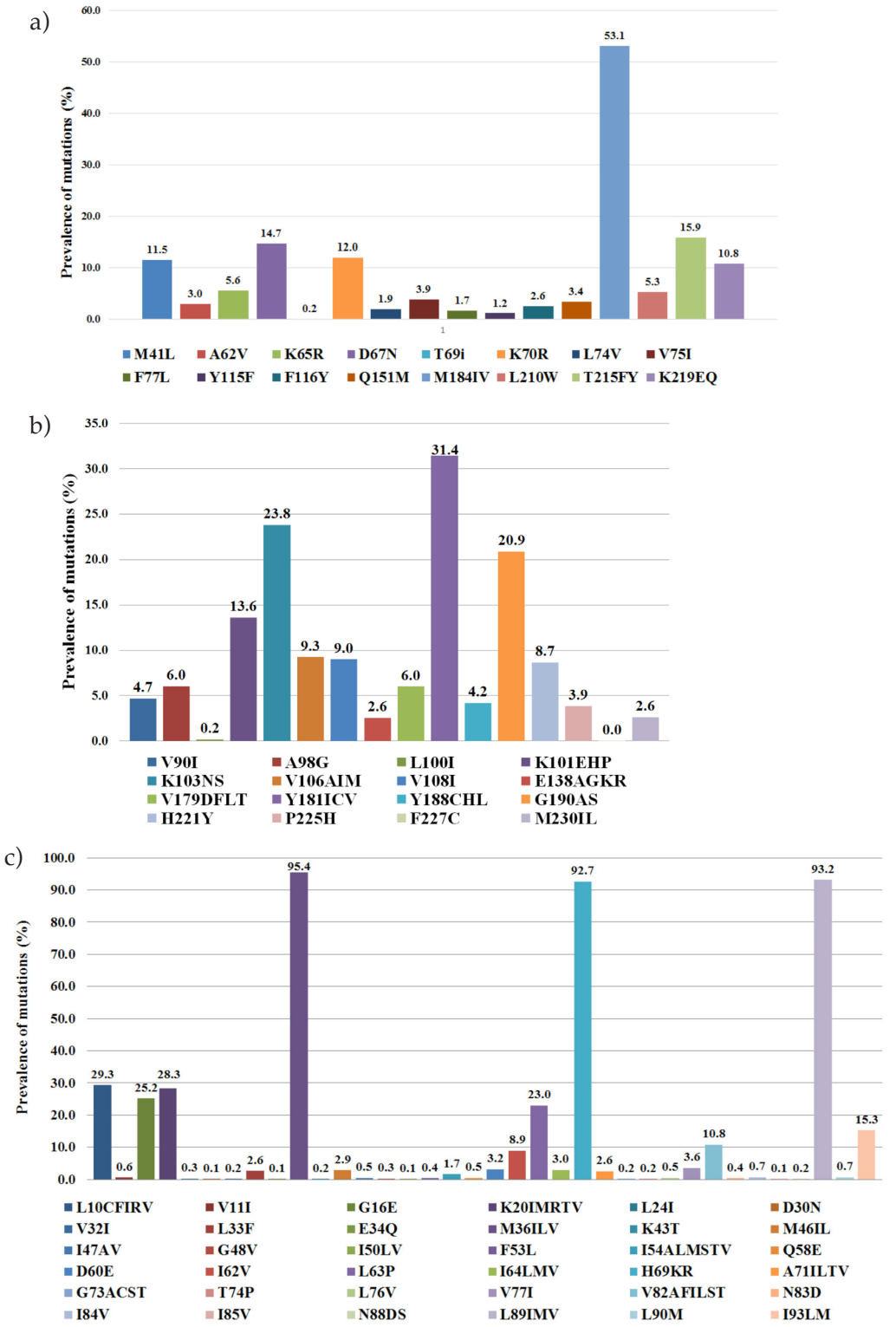

Fig 1–Prevalence of codon substitutions associated with resistance to antiretroviral drugs.

ing to 30% between 2000 and 2002, then climbed to 71.3% before decreasing to <50% in 2013 and 2014. The prevalence of other mutations (A62V, K65R, T69i, L74V, V75I, F77L, Y115F, F116Y, and Q151M) remained <10% from 1999 to 2014. However, a number of these mutations (A62V, K65R, T69i, L74V, V75I, F77L, Y115F, F116Y, and Q151M) slightly increased in prevalence between 2005 and 2008, then decreased.

The prevalence of resistant mutations to non-(N)NRTIs was low initially, then increased until 2007, when it remained constant thereafter (Fig 2b). Prevalence of most of the NNRTI mutations rose with time, except for K101E/H/P and F227C. Y181I/C/V prevalence initially at 5.9% in 1999 increased to 22.1-39.6 % from 2004 to 2007, before decreasing to slightly < 30% in 2013 and 2014. The prevalence of G190A/S also increased between 1999 and 2004 and remained at 20% after 2010. K103N prevalence increased during 1999 to 2004 then remained at 20-25% from 2006 to 2010, and increased after 2012. A98G, K101E/H/P, V108I and H221Y prevalence initially was < 10% between 1999 and 2003 before shifting to 10-15%, then decreased to < 10% after 2010, except that of K101E/H/P, which remained slightly > 10% after 2010.

Resistance-associated mutations in PR almost remained constant over the survey period (Fig 2c). M36I/L/V, H69K/R and L89I/M/V constituted 90% of the samples from 1999 to 2014, while L10C/F/I/R/V, K20IMRTV and L63P were presented 49.0%, 31.4%, and 33.3% in 1999, then remained around 20-30% afterwards. G16E prevalence was < 20% from 1999 to 2004, then rose to > 20% in 2007 and remained at around 25% until 2014. V82A/F/I/L/S/T was the most prevalent primary PI-resistant mutation found and together with I93L/M remained just >10% for over a decade, except during 2000, when they reached 22.6% and 23.7%, respectively. On the other hand, prevalence of I62V was 21.6% in 1999 before decreasing to 4.3% in 2000 and remaining >10% from 2001 to 2014.

Influence of Thai government HIV management policy on HIV-1 drug resistance

HIV-1 genotyping results of drug resistance mutations were analyzed in 4 periods, namely, 1999-2002 (introduction of antiretroviral drugs in Thailand), 2003-2006 (NAPHA launched), 2007-2010 [Universal Health Coverage (UC) scheme made available to HIV patients], and 2011-2014 (introduction of 2010 Thai National Guidelines) (Table 1). In 1999-2002, when antiretroviral drugs were made available, resistance to NRTIs was found in almost half (41.48%) of all samples and the proportion of resistance increased reaching a peak of 66.87% between 2007 and 2010, before decreasing to 51.71% in the fourth period ($p < 0.0001$, slope of the trend line = -0.0366). The proportion of highly resistant-NNRTI HIV-1 samples previously starting at a lower prevalence than that of NRTI-resistant HIV-1 at 29.69% from 1999 to 2002 increased dramatically to 63.5% and 70.87% in 2003-2006 and 2007-2010, respectively. Although NNRTI resistance decreased after those periods, it still remained >60% in 2011-2014 ($p < 0.0001$, slope = 0.0414). Unlike the other mutations, the proportion of PI resistance declines significantly from 14.03% in the first few years to <2% after 2006 ($p < 0.0001$, slope = -0.0211).

DISCUSSION

This study evaluated the prevalence of HIV-1 RT and PR drug-resistant mutations among chronically infected HIV-1 individuals in Thailand from 1999 to 2014. Most of the sequence data were obtained

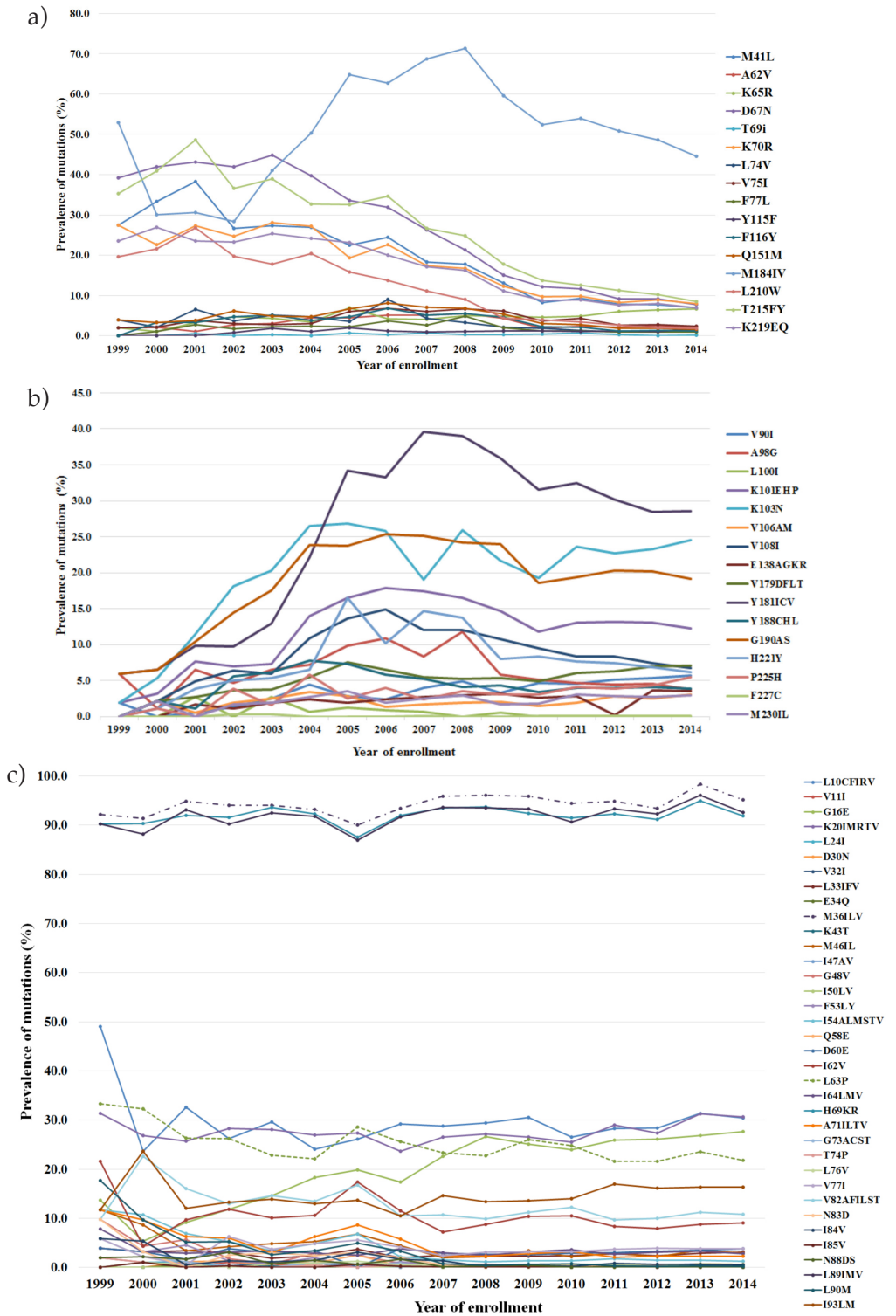

Fig 2–Time trends in frequency of antiretroviral resistant mutations in samples tested from 1999 to 2014. All mutations were associated with decreased susceptibility to NRTIs, NNRTIs and PIs.

Table 1
Chronically HIV-infected patients with drug resistance in Thailand from 1999 to 2014.

Drug	Resistance	1999-2002 (n = 687)	2003-2006 (n = 1,410)	2007-2010 (n = 8,168)	2011-2014 (n = 13,980)	p-value[a]
NRTI	Number (%)	285 (41.5)	781 (55.4)	5,462 (66.9)	7,229 (51.71)	< 0.0001
	95% CI	37.8 - 45.2	52.8 - 58.0	65.8 - 67.9	50.88 - 52.54	
NNRTI	Number (%)	204 (29.7)	896 (63.55)	5,789 (70.9)	9,330 (66.74)	< 0.0001
	95% CI	26.3 - 33.1	61.0 - 66.1	69.9 - 71.9	65.96 - 67.52	
		(n = 605)	(n = 999)	(n = 7,863)	(n = 13,509)	
PI	Number (%)	85 (14)	75 (7.5)	169 (2.1)	328 (2.43)	< 0.0001
	95% CI	11.3 - 16.8	5.9 - 9.1	1.8 - 2.5	2.17 - 2.69	

CI, confidence interval; NRTI, reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor. [a]Calculated chi-square test for linear trend to check a linear trend of drug resistance occurs in each period. A significant result suggests that the slope of the trend line is non-zero.

from patients registered for free AVRs under the UC scheme. In the years between 1999 and 2006, the UC scheme did not genotype HIV-1 free-of-charge, which resulted in a small number of samples available for this survey. However, NHSO began to provide free annual HIV-1 genotyping service from 2007, thereby significantly increasing the numbers of specimens.

In this study, M184V/I and NNRTI-resistant mutations were commonly detected because most of the samples came from patients who had failed an initial NNRTI-based regimen of lamivudine (3TC) and emtricitabine (FTC) (National Health Security Office, 2008; Sungkanuparph and Techasathit, 2011). The increase of M184V/I might have resulted from repeated use of 3TC or FTC in highly ARV-treated patients who already developed M184V/I. The goal of continual use of 3TC or FTC is for maintaining M184V/I HIV-1mutants to reduce viral fitness and delay the accumulation of zidovudine (AZT)-associated mutations in patients unable to switch their regimen to more expensive NRTIs (Miller *et al*, 2002). Although the presence of TAMs was found, their prevalence and the accumulation decreased continuously during the survey period. Apart from appropriate ART management, the reason that for the reduction of TAMs might have come from the change in medical practice since 2010 (Phanuphak *et al*, 2010). Stavudine (d4T) was no longer listed as a preferred regimen in Thailand due to its unwanted side effect (van der Valk *et al*, 2001; Pujari *et al*, 2005). The use of d4T gradually shifted to AZT that has less effect in generating TAMs, thereby leading to their decrease. Other NRTI resistance mutations, such as Q151M, conferring multiple-NRTI resistance and an indicator mutation for poor

ART management (Zaccarelli *et al*, 2004), was present at a very low level (< 10%), possibly due to the appropriate treatment plan during the past decade.

This survey revealed a long term increase of NNRTI resistance mutations associated with ARVs, which has been a backbone regimen in Thailand since 2004. Among the mutations, Y181I/C/V conferring cross-resistance to all NNRTI stood out. Fortunately, the spectrum of NNRTI resistance-associated mutations remained unchanged after 2007.

Certain mutations (*eg*, V90I) associated with resistance to second-line drug, etravirine (ETR), have been increasing during the UC scheme implemented since 2007. Bunupuradah *et al* (2011) reported high-level ETR resistance in HIV-1 subtype CRF01_AE-infected patients, who fail in the first regimen, but rare cases of resistance-associated mutations to rilpivirine (RVP), a second-generation NNRTI. Interestingly, our study found that cross-resistance mutations to RVP were present in Thailand since 2000. Furthermore, H221Y mutation, associated with RVP resistance only (Wensing *et al*, 2014), appeared in samples since 2000, although RVP was approved by the US Food and Drug Administration (FDA) in May 2011 (FDA, 2011) and has been used rarely in Thailand. The previous studies from Lambert-Niclot *et al* (2013) and Sungkanuparph *et al* (2013) also reported that H221Y could be found among RVP naïve patients. This demonstrates that using a new drug in previously anti-HIV treated and refractory patients in Thailand should be of concern regarding presence of pre-existing mutation(s) that could compromise the efficacy of the new drug.

It is noteworthy that approximately 90% of PI resistance-associated mutations were M36I/L/V, H69K/R and L89I/M/V, as well as L10C/F/I/R/V, K20I/M/R/T/V and L63P, which were found among samples that have been considered over decades as CRF01_AE polymorphisms (Kantor and Katzenstein, 2003; Manosuthi *et al*, 2010). Despite the fact that PR has a high genetic barrier against evolving mutations conferring resistance to PIs (Lee *et al*, 1998; Stoffler *et al*, 2002; Dierynck *et al*, 2007), our observations argue against the use of PI-based regimen as a first-line treatment (in addition to the high cost). Over the past years, the few patients who failed or could not tolerate HAART (2NRTI + 1NNRTI) regimen have receive a booster of PI regimen (Phanuphak *et al*, 2010; Sungkanuparph and Techasathit, 2011). This could explain why PI resistance-associated mutations were always present but at very low prevalence (< 20%).

The prevalence of HIV-1 drug resistance was influenced by the Thai government policy on HIV management, indicating that the policy regarding antiretroviral treatment could have played an influential role in ARV resistance prevalence. The rate of NRTI and NNRTI resistance among HIV patients increased from the start of HAART and accelerated in the period when the UC scheme became fully operative (2007-2010). Since then, the prevalence began to decline after the National Guideline was launched in 2010. This might indicate that, once the guidelines were followed, HIV treatment monitoring system, such as viral load measurement and HIV genotyping, allowed more effective drug treatment regimens, thereby reducing development of drug resistance.

It is important to note that these results may not represent resistance-associated mutation patterns in all HIV-1-infected individuals prescribed antiretroviral drugs.

Although the samples in this study were sufficient for obtaining a global picture of HIV-1 drug-resistant mutations in Thailand, they generally came from large centers. Virological tests were performed in other laboratory centers not included in the sample collection. Also, HIV-1 samples in this study mostly were CRF01_AE and may not represent virus populations from other demographic or geographic areas. This study focused only on HIV drug-resistant mutations in patients who met the criteria for HIV-1 drug resistance testing, *ie* having experience of ARVs and virological failure at a viral load > 2,000 copies/ml. Another limitation of the study was a lack of a control group for data comparison with antiretroviral-naïve patients. In addition, there is no pretreatment resistance testing as a conventional method for newly infected patients in the Thai UC scheme.

Future studies should include demographic characteristics together with treatment outcome to provide information on the association in HIV-1 drug resistance diversity under Thailand UC Scheme. According to a new guideline launched in early 2014 (Manosuthi *et al*, 2015), all patients should start ARV treatment without any specification of CD4 level. Thus, the mutation profile may change. Surveillance of HIV-1 resistant mutations should be continued to monitor emergence of resistant mutations in Thai patients who receive the new regimen. This will also allow detection of polymorphisms associated with resistance to new drugs, such as raltegravir.

In conclusion, this study illustrates how surveillance of HIV-1 dug resistance-associated mutations and HIV-1 drug resistance can be beneficial by providing an understanding of drug-specific mutation pattern in Thailand, where ARV program has been widely used. The results can act as key factors to evaluate effectiveness of the Universal Coverage Scheme in HIV treatment. Existing HIV drug resistance mutations should be taken into consideration before new antiviral drugs are added to the Thai national treatment policy.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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