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Development of a cost-effective assay for genotyping of HIV-1 non-B subtype for drug resistance

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Highly Active Antiretroviral Therapy (HAART) is the most effective way to control HIV-1 replication in infected patients. Prior to the start of therapy, genotyping of HIV-1 for mutations that confer resistance to potential drug candidates is crucial for it allows formulating an effective regimen. Ineffective drugs are excluded and potentially effective ones are included. A number of diagnostic kits are commercially available for this purpose but are tailored for HIV-1 subtype-B, a strain chiefly found in AIDS patients of Europe and America. However, AIDS patients of South-East Asia including Thailand are predominant infected with HIV-1 subtype non-B. In this study, an inexpensive assay was developed that genotypes HIV-1 non-B for drug resistance and tested it on 99 Thai AIDS patients. Results showed that 98 were infected with HIV-1 subtype non-B (or CRF01_AE) and one with subtype-B. Within the HIV-1 polymerase (*pol*), reverse transcriptase (RT) gene, the assay identified 18 codon mutations associated with resistance to Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) and 17 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs). Employing a commercially available kit, parallel genotyping of patient samples confirmed results providing validation of the assay. This method approximately costs 100 US dollars compared to $300 for a commercially available test. In Thailand, the burden of cost for treating HIV-infections is high not only for the average citizen but the country's health care systems. Therefore the low cost and yet effective genotyping test for HIV-1 subtype non-B is a practical and viable solution to expensive genotyping platforms.

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1. Introduction

Thailand's Department of Disease Control & Ministry of Public Health estimates that between 1984 and 2012 there were 276,947 people with AIDS and more than 1.2 million were infected with HIV (Bureau of Epidemiology, Thailand). Currently, Thailand has one of the highest HIV-1 infection rates among Asian countries which financially burdens government and private health care systems. The National Health Security Office (NHSO) and National Health Expenditure for HIV infection and AIDS reported that in 2010 and 2011 approximately 200 million US dollars was spent on AIDS related health care measures which include diagnostic testing and providing effective medication (National Health Security Office, Thailand).

Controlling the spread of HIV-1 among the masses is achieved through implementing safe health practices and marginalizing the viral reservoir in infected patients with effective therapy. It has been well established that antiretroviral therapies curb viral load and maintain adequate CD4+ T lymphocyte counts (Safren et al., 2005; Chitra et al., 2009; Manosuthi et al., 2009; Azzoni et al., 2011; Torti et al., 2011). Active replication in an infected patient can produce anywhere from 10^{10} to 10^{11} virions per day and given the high error rate of HIV-1 reverse transcriptase, there is a high probability that viral mutants resistant to a given drug/class of drugs arise (Preston et al., 1988; Hu and Temin, 1990; Holtz

Abbreviations: HAART, Highly Active Antiretroviral Therapy; NRTIs, Nucleoside/Nucleotide Reverse Transcriptase Inhibitors; NNRTIs, Non-Nucleoside Reverse Transcriptase Inhibitors.

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and Mansky, 2013). Furthermore drug resisting mutants can also result from genetic recombination mechanisms between two distinct proviral HIV-1 genomes within an infected cell (Hu and Temin, 1990). For these reasons a regiment of two or more antiretrovirals (HAART) has proven to an effective course for treating HIV-1 infections. Current HAART formulations encompass a spectrum of compounds that vary in their inhibition mechanism of viral replication. These include Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors, Fusion Inhibitors, and Integrase Inhibitors (Ammaranond and Sanguansittianan, 2012).

Identifying mutations in a patient's viral strain that can confer resistance to an antiviral or class of antivirals is the basis of a genotype based test. Existing commercial genotype kits assess the drug resistance of HIV-1 subtype B, a strain found in patients of Europe and America (UNAIDS). In Southeast Asia including Thailand patients are mostly infected with the recombinant form AE or non-B subtype (Manosuthi et al., 2013; Raymond et al., 2013; Sanguansittianant et al., 2013). Some studies have shown that commercial HIV-1 subtype B kits work well for various HIV-1 subtypes but with non-B subtypes results have not fared well (Fontaine et al., 2001; Ariyoshi et al., 2003; Beddows et al., 2003; Eshleman et al., 2004). In Thailand, a typical genotypic assessment for HIV-1 drug resistance costs a patient between 300 and 500 US dollars (National Health Security Office, Thailand), which is expensive for a citizen with average income. This combined with the lack of a sensitive test of HIV-1 non-B subtype has provided with the impetus for this work.

2. Materials and methods

2.1. Sample preparation and extraction of HIV-1 RNA

Peripheral blood from HIV-1 patients was provided by The Health Science Service Unit, HIV Laboratory, Faculty of Allied Health Sciences, Chulalongkorn University. Immediately upon collection (<6 h), blood in vacutainers (w/anticoagulant ethylenediaminetetraacetic acid, EDTA) was centrifuged at 1500 rpm for 10 min, 1 ml of plasma was removed for HIV RNA quantification with COBAS® Taqman® HIV-1 test (Roche Molecular Diagnostics, Switzerland) and 1 ml of plasma was stored in sterile cryovials at −70 °C for RNA extraction.

Cryovials with frozen plasma were rapidly thawed and 650 μl of plasma with over 2000 copies/ml of viral load, was extracted with COBAS® AmpliPrep Total Nucleic Acid Isolation kit (Roche Molecular Diagnostics) and genotyped by in-house test in parallel with the commercial kit Trugene HIV Genotyping test (Siemens Healthcare Diagnostics, Australia).

2.2. Reverse transcription of patient HIV-1 RNA into cDNA

Fig. 1 flow charts the strategy of in-house assay. To confine the genotype test to HIV-1 non-B, 4 primers were designed to amplify relevant regions of the polymerase (*pol*) gene of CM240, an HIV-1 strain found predominately in Thailand's AIDS patients (GenBank no. AF447851.1):

1. RT-PCR-end (5′-GCTCCCCTGAGGAGTTTACACA-3),
2. PA3 (5′-GGAATTTTCCTCAGAGCAGACCAG-3′),
3. PA1 (5′-GCTTTACCTTAATCCCTGCATAAA-3′) and
4. RT-PCR-start (5′-TTAGTAGGACCTACACCTGTCAACAT-3′).

HIV-1 RNA in extracted samples was reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT, Promega, USA). The reaction was carried out by placing 10 μl of RNA into PCR tubes, heated to 70 °C (~5 min) to denature its secondary structure, cooled (on ice for ~5 min), mixed with 15 μl of RT-PCR master mix and thermocycled (GeneAmp, USA) for one round of reverse transcription; 42 °C for 45 min, 95 °C for 5 min, 4 °C hold and all ramp times were 1 °C/s. Per-reaction, the master mix consisted of 5.0 μl of 5xMMLV RT buffer, 2.0 μl of 25 mM $MgCl_2$, 2.0 μl of 2.5 nM dNTP, 1.0 μl of 10 ρmole/μl RT-PCR-end primers, 4.25 μl of distilled water, 0.5 μl of 200 U/μl M-MLV RT enzyme and 0.25 μl of 40 U/μl RNase-Inhibitor.

2.3. Amplification of transcribed cDNA

HIV viral genome was amplified on *pol*. For standard PCR method, 10 μl of the transcribed cDNA was transferred into PCR tubes containing 40 μl of master mix and thermocycled for one cycle at 94 °C for 4 min, 35 cycles at (94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min) and one cycle at 72 °C for 7 min with a 4 °C hold. All ramp times were at 1 °C/s. Master mix contained per reaction: 5.0 μl of 10xTaq buffer, 2.0 μl of 25 mM $MgCl_2$, 4.0 μl of 2.5 mM dNTP, 1.5 μl of 10 ρmole/μl RT-PCR-end primers, 1.5 μl of 10 ρmole/μl of PA3 primers, 25.5 μl of distilled water, and 0.5 μl of 5 U/μl of Taq DNA polymerase (Thermo Fisher Scientific, EU).

For nested PCR technique, 5 μl of PCR product from the above amplification in PCR tubes was mixed with 45 μl of master mix and thermocycled with the same settings of standard PCR method as above. Nested PCR master mix contained per reaction: 5.0 μL of 10xTaq buffer, 2.0 μl of 25 mM $MgCl_2$, 4.0 μl of 2.5 mM dNTP, 1.5 μl of 10 ρmole/μl RT-PCR-start primers, 1.5 μl of 10 ρmole/μl PA1 primers, 30.5 μl of distilled water and 0.5 μl of 5 U/μl Taq DNA polymerase (Thermo Fisher Scientific, EU). The expected size of PCR product of 904 bp was confirmed by 0.7% agarose gel electrophoresis/UV lamp detection with 100 bp DNA markers (Fermentas, Thermo Fisher Scientific, EU). The final PCR product was purified by HiYield™ Gel/PCR Fragments Extraction Kit (RBCBioscience, Taiwan).

2.4. Sequencing of the amplified cDNA pol-RT gene

The amplified cDNA was sequenced employing 6 primers that specifically tailored to target expected mutations within the HIV-1 reverse transcriptase region. The primers were of 20–30 bases long providing ideal melting temperature and annealing times for standard PCR reactions. These primers (listed below) were employed by 1st BASE DNA Sequencing Services (Malaysia) to sequence the HIV-1 *pol*-RT gene extracted and amplified from patient samples.

1. RT41 (5′-CCCAATTAGTCCTATTGACACTGTA-3′),
2. RT74 (5′-GATGCGGTATTCCTAATTGAACTTC-3′),
3. RT100 (5′-GGCCTGAAAATCCATACAATACTCC-3′),
4. RT108 (5′-GTACTGATATCTGATTCCTGGTGTCTCA-3′),
5. RT181 (5′-CAATGTGCTGCCACAGGGATG-3′) and
6. RT219 (5′-CAGCTGTCTTTTTCTGGCAGTTCTA-3′).

For sequencing HIV-1 RNA samples with Trugene HIV Genotyping Kit, standard operating procedures of the vendor were closely followed. In brief, cDNA of patient samples were PCR amplified for HIV-1 *pol* genes (297 and 1680 nucleotides in length), vendor primers targeted protease and reverse transcriptase regions. The amplified fragments were sequenced by a "CLIP" reaction that employed fluorescently labeled forward and reverse primers. Upon completing of the reaction fluorescent fragments were detected by polyacrylamide gel electrophoresis using Long-Read Tower Sequencer (Siemens Healthcare Diagnostics, Australia).

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