Journal of Virological Methods 217 (2015) 70–78

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

An effective tool for identifying HIV-1 subtypes B, C, CRF01_AE, their recombinant forms, and dual infections in Southeast Asia by the multi-region subtype specific PCR (MSSP) assay

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Article history:
Received 8 August 2014
Received in revised form 5 February 2015
Accepted 14 February 2015
Available online 25 February 2015

Keywords:
HIV-1
Subtyping
MSSP assay
Subtype specific PCR
Southeast Asia

A B S T R A C T

The RV144 Thai vaccine trial has been the only vaccine study to show efficacy in preventing HIV infection. Ongoing molecular surveillance of HIV-1 in Southeast Asia is vital for vaccine development and evaluation. In this study a novel tool, the multi-region subtype specific PCR (MSSP) assay, that was able to identify subtypes B, C, CRF01_AE for Thailand, other Southeast Asian countries, India and China is described. The MSSP assay is based on a nested PCR strategy and amplifies eight short regions distributed along the HIV-1 genome using subtype-specific primers. A panel of 41 clinical DNA samples obtained primarily from opiate users in northern Thailand was used to test the assay performance. The MSSP assay provided 73–100% sensitivity and 100% specificity for the three subtypes in each genome region. The assay was then field-tested on 337 sera from HIV infected northern Thai drug users collected between 1999 and 2002. Subtype distribution was CRF01_AE 77.4% (n = 261), subtype B 3.3% (n = 11), CRF01_AE/B recombinant 12.2% (n = 41), CRF01_AE/C recombinant 0.6% (n = 2), and non-typeable 6.5% (n = 22). The MSSP assay is a simple, cost-effective, and accurate genotyping tool for laboratory settings with limited resources and is sensitive enough to capture the recombinant genomes and dual infections.

1. Introduction

A Thai epidemic of HIV-1 subtype B and CRF01_AE was identified in 1990 (Weniger et al., 1991; McCutchan et al., 1992). Recombination between these strains was identified in 1997 (Tovanabutra et al., 2001), and recombinant strains, including additional 3 circulating recombinant forms or CRFs:, CRF15_01B, CRF34_01B and CRF52_01B, have been found in many Thai cohorts (Viputtikul et al., 2002; Tovanabutra et al., 2003, 2007; Liu et al., 2012). Within the region, a recombinant of CRF01_AE and subtype C was reported in Vietnamese intravenous drug users (Tran et al., 2004), and recombination among CRF01_AE, B′ and C was described in northern Myanmar (Pang et al., 2012). Series of CRFs between CRF01_AE and subtype B were identified in Malaysia, Singapore and China (CRF33_01B (Tee et al., 2006), CRF48_01B (Li et al., 2010), CRF51_01B (Ng et al., 2012b), CRF53_01B (Chow et al., 2012), CRF54_01B (Ng et al., 2012a), and CRF55_01B (Han et al., 2013)). In China subtypes B, C, CRF01_AE, CRF07_BC, CRF08_BC co-circulate and their intersubtype recombinants have emerged in Southeast Asia (Liu et al., 2011; Chen et al., 2013; Su et al., 2013).

Abbreviations: CRFs, circulating recombinant forms; HIV-1, human immunodeficiency virus type 1; HMA, heteroduplex mobility assay; MHA, multi-region hybridization assay; MSSP, multi-region subtype specific PCR; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; Tm, melting temperature; V3-PEIA, V3 loop-peptide ELISA.

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http://dx.doi.org/10.1016/j.jviromet.2015.02.015
0166-0934/

The Thai HIV-1 vaccine trial RV144 showed modest protection against HIV infection (Rerks-Ngarm et al., 2009), and additional studies to confirm and extend RV144 are planned in Thailand. Hence, ongoing molecular surveillance of HIV-1 in Thailand is vital for vaccine development and evaluation. Considering the evolving complexity of the HIV-1 epidemic in and around Thailand, monitoring of the HIV-1 genetic subtypes circulating in these countries is also important. Therefore, a simple and rapid tool for screening circulating genetic subtypes and their recombinant forms is needed for HIV-1 genetic surveillance.

Assays for HIV-1 subtyping include the heteroduplex mobility assay (HMA) (Delwart et al., 1993, 1995; Bredell et al., 2000; Heyndrickx et al., 2000; Tatt et al., 2000), V3 loop-peptide ELISA (V3-PEIA) (Gaywee et al., 1996; Hoelscher et al., 1998), subtype-specific PCR in *vpu*, *env*, and *gag* region (Chen et al., 2002; Yagyu et al., 2002; Luo et al., 2011), restriction fragment length polymorphism (RFLP) (van Harmelen et al., 1999), and a single nested multiplex PCR (Gomez-Carrillo et al., 2004). A major limiting factor of these tools is that they cannot identify recombinant strains and dual infections accurately because only parts of one or two genes are examined. The multi-region hybridization assay (MHA) has been developed and used effectively to distinguish HIV-1 subtype and recombinant forms in various epidemic areas including Thailand (Hoelscher et al., 2002; Watanaveeradej et al., 2006; Kijak et al., 2007). However, MHA can only be performed in laboratories with real-time PCR capability, and interpretation of the data requires significant training and experience.

In this study, the same strategy used in MHA assay to screen genetic subtypes of HIV-1 across the genome was applied to develop a new multi-region subtype specific PCR assay (MSSP), in a format that can be performed in resource limited setting. The multi-region subtype specific PCR assay has been developed and evaluated to be used as a tool for identifying HIV-1 subtypes B, C, and CRF01_AE, and their recombinant viruses circulating in Thailand and neighboring countries. It is a PCR-based method to differentiate genetic subtype in 8 regions across HIV-1 genome: *Gag (p17-p24), Pol (p2-p7-p6 protease), Pol (p51 RT), Pol (p31 integrase), Vpr, Env (gp120), Rev, and Nef.*

This new assay is different from MHA assay because subtype specific primers, instead of probes, have been used in the assay, and the detection is based on gel electrophoresis, instead of real-time PCR. The MSSP has been evaluated on a panel of 41 clinical samples characterized previously by full genome sequencing and then field tested on 337 HIV-1 prevalent samples collected from injecting drug users in northern Thailand.

2. Materials and methods

2.1. Sample collections and DNA/RNA extraction

DNA of known full genome HIV-1 sequences were used as PCR templates in the initial test to evaluate the new assay's sensitivity and subtype specificity. The strains used were CRF01_AE: 02TH.OUR737I (AY358037) and 99TH.OUR199I (AY358039), co-cultured PBMC DNA of subtype B: 96TH_NP1538 (AY713408) and NPBQC (KJ769147) and near full-length genome cloned of subtype C: 95IN21068 (AF067155) and 93IN905 (AF067158) obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Primary PBMC DNA was used for the evaluation of clinical samples, which came from a panel of 41 DNA samples including CRF01_AE ($n = 33$), CRF15_01B ($n = 2$) (Tovanabutra et al., 2003), and unique CRF01_AE/B recombinants ($n = 6$). Three of the clinical samples were prevalent cases and the other 38 were incident cases (Tovanabutra et al., 2004) from a study of northern Thai intravenous drug users (Razak et al., 2003). The DNAs were extracted from peripheral blood mononuclear cells (PBMCs) using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

Blood samples were also obtained from the a study of northern Thai intravenous drug users or OUR cohort collected between 1999 and 2002 at the Northern Drug Treatment Center (NDTC) in Chiang Mai, Thailand (Razak et al., 2003). There were 1865 volunteers (1665 men and 200 women) that completed the study procedures. The overall HIV prevalence was 10.3%. The prevalence was 30.0% among 513 injection drug users, and 2.8% among 1352 non-intravenous drug users (OR = 14.8, 95% CI: 10.2, 21.6). The study was extended to recruit an additional 366 intravenous drug users, and 42.4% ($n = 155$) of them were HIV positive. Ultimately, 337 serum samples from a total of 347 HIV-1 infected individuals were available for genetic subtyping (Razak et al., 2003). Serum viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Subtype distributions using MHA genotyping, demographic characteristics and risk factors of this study population were described elsewhere (Kijak et al., 2011).

2.2. Primer design and MSSP assay development

In the MSSP assay 7 regions of the HIV-1 genome were amplified in the first round PCR, and 8 regions were amplified in the second round. The first round PCR was performed using universal primers that enabled amplification of all subtypes in *p17-p24, p2-p7-p6 protease, p51 RT, p31 integrase, vpr, gp120, rev*, and *nef*. Then, each first round PCR product was divided to perform three reactions in second round PCR with different subtype specific 5′ and 3′ primers. One first round amplicon encompassing the *rev* and *nef* genes was utilized by subtype specific primers for both *rev* and *nef*. The locations of PCR amplicons of the MSSP assay on HIV-1 genomic structure are shown in Fig. 1.

To select the HIV-1 genome regions that allow for the differentiation of subtypes B, C and CRF01_AE, a database of 71 near full-length genome sequences from the HIV strains isolated in Thailand and the neighboring countries were downloaded from GenBank and aligned. Recombination breakpoints in the genome structures of 16 CRFs and URFs identified recently in Thailand and neighboring countries were mapped (Fig. 2). Regions of interest where the nucleotides were highly conserved intraclade and highly diverse interclade were selected across HIV genome. Outer primers were designed to locate on highly conserved regions between subtypes in order to amplify all of the three subtypes (B, C, and CRF01_AE). Inner primers were subtype-specific primers and located at highly divergent regions between these three subtypes. These primers were designed to incorporate multiple mutations distributed along their sequences. The melting temperatures (Tm) of these primers were approximately 60 °C. Primer-dimer and hairpin loop formation had ΔG of more than −6.0, and −1.5 kcal/mol, respectively.

2.3. PCR conditions

For the DNA template, the first round MSSP contained 2 μl of genomic DNA, 200 μM each dNTP, 1.5 mM $MgCl_2$, 400 nM primers, 1.05 U AmpliTaq GOLD in 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) in a final volume of 21 μl. The second round PCR contained 1 μl of diluted 1 in 10 of first round PCR products, 200 μM each dNTP, 1.5 mM $MgCl_2$, 400 nM primers, 0.55 U AmpliTaq GOLD in 1× PCR buffer (Applied Biosystems, Foster City, CA, USA) in a final volume of 11 μl.

For the RNA template, the first round RT-MSSP was performed by one step RT-PCR (Qiagen, Hilden, Germany). The reaction contained 2.5 μl of extracted viral RNA, 400 μM each dNTP, 800 nM primers, and 1 μl enzyme mix in a 25 μl total reaction with 1X