

RESEARCH NOTE

USE OF DRIED BLOOD SPOTS FOR HIV-1 GENOTYPING IN SOUTHEAST ASIA: THAILAND EXPERIENCE

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Abstract. The multi-region hybridization assay (MHAbce) for genotyping HIV-1 subtypes B, C and circulating recombinant form (CRF01_AE) was evaluated on paired plasma and dried blood spots (DBS) collected from 68 HIV-1 infected individuals in Thailand. CRF01_AE was the predominant subtype identified using plasma samples (51/62) and DBS (24/27). There was no discordance in subtype designations between plasma and DBS.

Keywords: HIV subtyping, dried blood spot

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INTRODUCTION

One of the greatest challenges facing development of an effective HIV-AIDS vaccine is the extensive sequence diversity of the virus. HIV-1 genotypes surveillance in remote regions of the world is often problematic due to the lack of the necessary infrastructure to process specimens from phlebotomy tubes and maintenance of cold facilities for specimen storage and transport (Quinn *et al*, 1986; Tovanabutra *et al*, 2004b). To overcome these difficulties, dried blood spots (DBS) collected on filter papers have been used for HIV-1 serology and molecular studies as this simplifies specimen processing, storage and transportation (Farzadegan *et al*, 1987; Lindhardt *et al*, 1987; Leelawiwat *et al*, 2009). In addition, the use of DBS is more economical than that of plasma for sample preparation and archiving as

venipuncture is not required.

Several assays for HIV-1 diagnostics, viral load and subtyping have been successfully adapted for use with DBS. Serologic HIV-1 diagnosis from DBS demonstrated equivalent sensitivity to traditional serum and plasma testing (Farzadegan *et al*, 1987; Lindhardt *et al*, 1987). HIV-1 DNA obtained from DBS correlated with RNA viral load from plasma and whole blood (Leelawiwat *et al*, 2009). HIV-1 drug resistance profiles as well as DNA sequencing of the *rt* and *pr* regions could be performed on DNA extracted from DBS (Steegen *et al*, 2007). HIV-1 subtypes have been assigned from DBS using genetic sequencing (Cassol *et al*, 1996) and serotyping (Chanbancherd *et al*, 1999). In addition, drug resistance profiles and diverse HIV-1 genotyping of the HIV-1 *pol* region have been achieved using DBS collected from diverse geographic regions (Yang *et al*, 2010).

The extensive sequence diversity of HIV-1 and emergence of novel, circulating recombinant forms (CRF) require ongoing surveillance of HIV-1 (Altfeld *et al*, 2002; Perez-Alvarez *et al*, 2002; Ramos *et al*, 2002; Thomson *et al*, 2002; Tovana-butra *et al*, 2004a,b). Therefore, an HIV-1 genotyping assay based on multiple regions of the genome facilitated a proper identification of viral stains, helping to characterize transmission within populations and to potentially contribute in HIV-1 vaccine design. In Southeast Asia, the predominant circulating HIV-1 strain is CRF01_AE, with minor representation of subtypes B and C also being reported from Thailand (Tovanabutra *et al*, 2004 a,b). A multi-region hybridization assay (MHAbce) for characterization of HIV-1 subtypes B, C and CRF01_AE by quantitative PCR utilizing probes in 8 regions

throughout the viral genome for higher resolution genotyping has been developed (Hoelscher *et al*, 2002; Kijak *et al*, 2007). An HIV-1 genotype was assigned when the subtype-specific probes react with ≥ 4 out of 8 regions. In order to extend and improve HIV-1 genotype surveillance efforts in remote areas that lack the necessary laboratory infrastructure for specimen processing, we investigated the feasibility of using MHAbce on DBS for HIV-1 genotyping of samples from the Southeast Asian region.

MATERIALS AND METHODS

We evaluated the utility of DBS-MHAbce in the previously validated plasma-MHAbce of 68 pairs of matched plasma (200 μ l of plasma per subject) and DBS (80 μ l whole blood per subject) collected on 903 Protein Saver cards (Whatman, Maidstone, UK) and air dried at room temperature and stored at -20°C until testing from anti-retroviral-naive, HIV-1 infected Thai volunteers with different plasma HIV-1 viral loads (Table 1). Total DNA was extracted from DBS using QIAamp DNA Blood Mini Kit Spin (QIAGEN, Valencia, CA). DNA was quantified using NanoDrop (Thermo, Wilmington, DE) and gel visualization. HIV-1 viral RNA was extracted from plasma using QIAamp MinElute Virus Kit (QIAGEN, Valencia, CA) in accordance with the manufacturer's instructions. IRB approval was obtained in both Thailand and the USA prior to starting the study.

MHAbce analysis was performed on the extracted DNA and RNA as previously described (Kijak *et al*, 2007). In brief, extracted RNA was reverse transcribed, followed by PCR amplification using QIAGEN OneStep RT-PCR kit

Table 1
Relationship between HIV-1 viral load and MHAbce sensitivity from matched plasma and DBS samples.

HIV-1 viral load (copies/ml)	No. of samples	No. of PCR positive samples ^a		No. of samples genotyped ^b	
		Plasma-MHAbce (%)	DBS-MHAbce (%)	Plasma-MHAbce (%)	DBS-MHAbce (%)
0-99	2	1 (50)	0	0	0
100-499 ^c	3	3 (100)	0	3 (100)	0
500-999	2	2 (100)	0	2 (100)	0
1,000-4,999	18	18 (100)	6 (33)	18 (100)	4 (67)
5,000-9,999	10	10 (100)	2 (20)	9 (90)	2 (100)
10,000-49,999	18	18 (100)	15 (83)	16 (89)	8 (53)
50,000-99,999 ^d	7	7 (100)	7 (100)	7 (100)	7 (100)
100,000-≥499,999	8	8 (100)	8 (100)	7 (88)	6 (75)

^aNumber of PCR positive reactions obtained for at least 4/8 regions; (%), number PCR positive samples/total samples. ^bNumber of samples that have probes reactive with at least 4/8 regions; (%), number probe reactive samples/PCR positive samples/total samples. ^cEstimated sensitivity of plasma-MHAbce: 100 copies/ml (100% successful rate for PCR sensitivity). ^dEstimated sensitivity of DBS-MHAbce: 50,000 copies/ml or 4,000 copies/DBS (100% successful rate for PCR sensitivity).

Table 2
Comparison of plasma and DBS-MHAbce performance.

Analysis	MHA evaluation	Overall performance (%)	HIV-1 genome regions ^a (%)							
			<i>p17</i>	<i>pr</i>	<i>rt</i>	<i>int</i>	<i>tat</i>	<i>gpl20</i>	<i>gp41</i>	<i>nef</i>
PCR positive ^{b,c}	Plasma	522/544(96)	67/68(99)	66/68(97)	66/68(97)	67/68(99)	66/68(97)	65/68(96)	62/68(91)	63/68(93)
	DBS	266/544(49)	28/68(41)	32/68(47)	35/68(51)	40/68(59)	26/68(38)	34/68(50)	36/68(53)	35/68(51)
Probe positive ^d	Plasma	416/522(80)	54/67(81)	39/66(59)	60/66(91)	62/67(93)	59/66(89)	50/65(77)	48/62(77)	44/63(70)
	DBS	207/266(78)	24/28(86)	25/32(78)	33/35(94)	32/40(80)	22/26(85)	26/34(76)	24/36(67)	21/35(60)

^aEight regions of HIV-1 genome analyzed by either plasma or DBS-MHAbce. ^bNumber of PCR positive reactions based on Syber Green Pro file. ^c(%), Number of PCR positive reactions/total number of PCR reactions. ^d(%), Number of probe positive reactions/total number of PCR positive reactions.

(QIAGEN, Valencia, CA). Each PCR amplicon was subjected to 8 second-round PCR reactions, each using a different fluorescent subtype-specific probe in a TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA), and to a quantitative PCR containing TaqMan® Syber Green Mix (Applied Biosystems, Foster City, CA) to determine whether sample amplification had occurred. A specific HIV-1 subtype was assigned when probes reacted with $\geq 4/8$ regions.

RESULTS

Sensitivity of DBS-MHAbce was estimated by conducting MHAbce on DBS containing discarded HIV-1 sero-negative blood spiked with serial dilutions (10-100,000 copies/ml) of the ACH2 cell line, which carries a single integrated copy of subtype B HIV-1 DNA per cell (Clouse *et al.*, 1989). Beta-globin was utilized as an internal standard for DNA loading prior to conducting the MHAbce assay. The results showed that the sensitivity (defined as 100% successful rate for obtaining PCR positive reactions ≥ 4 out of 8 regions) of the DBS-MHAbce was 50,000 copies of HIV-1 DNA/ml of whole blood or 4,000 copies HIV-1 DNA/80 μ l of DBS (data not shown). For the paired plasma and DBS samples, 15/68 contained viral load of $\geq 50,000$ copies/ml of plasma. When plasma viral load was $\geq 50,000$ copies/ml, 15/15 PCR positive amplifications were observed (Table 1). Whereas in DBS, 13/15 samples (87%) with viral load $\geq 50,000$ copies/ml and 14/53 (26%) with viral load $< 50,000$ copies/ml ($p < 0.01$; Fisher's exact test) were genotyped successfully by MHAbce.

When the overall performance of DBS-MHAbce was compared to that

of plasma-MHAbce, using total of 544 MHAbce reactions (68 samples \times 8 regions), that of DBS-MHAbce showed a lower overall PCR amplification frequency (49%) but comparable probe reactivity (78%) in all regions compared to plasma (80%) (Table 2). The frequency of PCR amplification in the 8 regions throughout the HIV-1 genome ranged from 62 - 67 of 68 (91 - 99%) for plasma and 26 - 40 of 68 (38 - 59%) for DBS, likely due to the lower volume of specimen in the latter. In addition, probe sensitivity in the 8 regions of the HIV-1 genome for plasma- and DBS-MHAbce varied from 59 - 93% (39/66 and 62/67) and 60 - 94% (21/35 and 33/35), respectively (Table 2). However, the MHAbce probes showed comparable performance between plasma and DBS regardless of subtype once there were adequate regions of the HIV-1 genome amplified to perform the assay (Table 3).

A total of 62/67 (93%) samples were genotyped using plasma compared with 27/38 (71%) for DBS (Table 3). A total of 5/67 (7%) plasma *versus* 11/38 (29%) DBS were non-typeable by MHAbce ($p < 0.01$). The 62 samples assigned a genotype using plasma represented HIV-1 infections with CRF01_AE (82%), subtype B (6%) and others (11%). Despite the PCR amplification of only 38/68 (56%) DBS samples allowing MHA genotyping compared to 67/68 (99%) of plasma samples, the overall HIV-1 subtype distributions were comparable: CRF01_AE, (89% *versus* 82%); subtype B, (7% for both specimen types); and others, (4% *versus* 11%).

Dual probe infections were detected in 1/27 (4%) of the genotyped DBS samples in comparison to 2/62 (3%) of the genotyped plasma samples. The subtype assignments using DBS were concordant with those using plasma (Table 4).

Table 3
Comparison of plasma and DBS-MHAbce genotype distributions.

Sample source	Total	No. of samples used for MHAbce ^a (%)	No. of samples genotyped by MHAbce ^b (%)	Genotypes (%)			
				CRF01_AE	B	Other ^c	NT ^d
Plasma	68	67 (99) ^e	62 (93)	51 (82)	4 (6)	7 (11)	5 (7)
DBS	68	38 (56) ^f	27 (71) ^f	24 (89)	2 (7)	1 (4)	11 (29) ^f

^aPCR positive reactions obtained for at least 4/8 analyzed regions.

^bNumber of samples genotyped by MHAbce; (%), No. of samples genotyped by MHAbce/No. of samples used for MHAbce.

^cOthers defined as either CRF01_AE/B recombinant or CRF01_AE/B dual infection.

^dNon-typeable defined as samples with probe reactivity in less than 4 regions.

^eNumber of samples that have probes reactive with at least 4 regions.

^f $p < 0.01$ compared to plasma.

Table 4
Concordance of MHAbce genotype assignment between plasma and DBS.

DBS	Plasma		
	B	CRF01_AE	Others ^b
B	2 ^a	0	0
CRF01_AE	0	24	0
Others ^b	0	0	1
NT ^c	2	27	6

^aNumber of samples.

^bEither CRF01_AE/B recombinant or CRF01_AE/B dual infection.

^cNon-typeable due to either un-amplifiable or insufficient probe reactivity for subtyping ($N < 4$).

DISCUSSION

The results from our study agreed with other observations that CRF01_AE is the predominant form of HIV-1 circulating in Thailand (Tovanabutra *et al*, 2004 a,b; Kijak *et al*, 2007; Arroyo *et al*, 2010). The major limitation of our study was the limited sensitivity of the DBS genotyping method at a viral load below 50,000 copies/ml of blood. Plasma allowed a 2-fold higher number of samples to be

genotyped by the MHAbce compared to DBS, due to the larger sample volume used as template. Ongoing studies in our laboratory are focusing on optimizing the DNA extraction from DBS.

The performance of the DBS-MHAbce demonstrated a lower PCR sensitivity than plasma-based genotyping, limiting the number of regions that could potentially react to one of the HIV-1 subtype specific probes used in MHA. The overall

subtype distribution based on the DBS was similar when compared to the previously validated plasma specimens despite the lower PCR sensitivity of the former (Kijak *et al*, 2007; Arroyo *et al*, 2010). The result suggests that the DBS-MHAbce provided a similar HIV-1 subtype distribution. The DBS-MHAbce technique could be a practical tool for characterizing the predominant HIV-1 strains in remotes areas in Asia but the assay requires further refinement, with particular emphasis on improving the efficiency in extracting HIV-1 DNA from DBS, thereby increasing viral template amount and assay sensitivity.

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