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Application of Dried Blood Spot Specimens for Serologic Subtyping of Human Immunodeficiency Virus Type 1 in Thailand

P. CHANBANCHERD,[1] A. E. BROWN,[2] R. TRICHAVAROJ,[2] P. TIENAMPORN,[1] P. PUTHAKIRD,[1] N. LIMPAIROJN,[1] T. C. VANCOTT,[3] AND M. S. DE SOUZA[2,4]*

Army Institute of Pathology,[1] Armed Forces Research Institute for Medical Sciences,[2] and Henry M. Jackson Foundation for the Advancement of Military Medicine,[4] Bangkok, Thailand, and Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, Maryland[3]

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Dried blood spot (DBS) specimens were assessed as an alternative to plasma for human immunodeficiency virus type 1 (HIV-1) serotyping by V3 loop peptide enzyme immunoassay. Nested PCR capable of distinguishing HIV-1 subtypes B and E was used as the reference standard. Ninety-two percent of DBS samples were typeable as either HIV-1 subtype B or E. Serotype results with DBS and plasma were identical for 254 of 257 specimens. A simple DBS collection method provides a convenient alternative for conducting HIV-1 serotype surveillance while retaining sensitivity and specificity.

The human immunodeficiency virus type 1 (HIV-1) epidemic in Southeast Asia occurred relatively late compared to that in Europe (4), but the epidemic has been explosive, and in certain areas multiple subtypes are cocirculating (11). Thailand was selected by the World Health Organization as a major site for HIV-1 vaccine trials. One of the problems of the HIV-1 pandemic is the existence of multiple subtypes or clades, both within and between geographic regions, which may affect vaccine strategies. Monitoring of the circulating strain or subtype is also important in tracking the spread and trend of an HIV-1 epidemic. Dried blood spot (DBS) specimens have been used in screening antibodies to measles virus and, more recently, antibodies to HIV-1 (5, 8). In Southeast Asia and many developing countries, the expense involved in the use of traditional venipuncture and vacutainer serum or plasma separation tubes, as well as difficulties in transportation to the laboratory and the limited shelf life of serum and plasma outside of refrigerated storage, can hinder large-scale HIV-1 surveillance.

Subtyping of HIV-1 can be conducted by either genetic or serologic approaches. Genetic subtyping, while the most definitive method, is extremely costly and requires relatively elaborate specimen collection, processing, and technical expertise. Since the V3 loop peptide enzyme immunoassay (V3-PEIA) was introduced for preliminary HIV-1 subtyping, success has been reported with HIV-1 subtypes A, B, C, and E. In Thailand, two clades of HIV-1, subtypes B and E, are currently circulating. V3-PEIA has been used in that country for serotyping antibody responses to these subtypes with high specificity (95 to 100%) and sensitivity (85 to 95%) (9).

In this study, V3 serotyping of antibodies eluted from DBS was compared with that of plasma antibodies, and both methods were compared with PCR-based genotyping of HIV DNA from the same individuals. Three milliliters of whole blood was collected from 257 HIV-1-seropositive and 8 HIV-1-seronegative subjects, based on screening with a gel particle agglutination assay (Serodia-HIV; Fujirebio, Tokyo, Japan) and confirmation by Western blotting (Bio-Rad, Hercules, Calif.). Blood was collected with potassium-EDTA vacutainer tubes (Becton-Dickinson, Franklin Lakes, N.J.), divided into three aliquots, and treated as follows. Duplicates of 5 and 20 μl of whole blood were spotted onto the circled areas of standard filter paper (no. 903; Schleicher and Schuell, Keene, N.H.). Samples were air dried at room temperature (25 to 35°C) for a minimum of 18 h and placed in individual Ziploc bags and stored at room temperature in the dark until testing. Approximately 1 ml of blood was centrifuged at 800 × *g* for 10 min; the plasma layer was removed and stored at −20°C until testing. The remaining blood (approximately 1 ml) was treated with 3 ml of FACScan lysing solution (Becton-Dickinson) for 10 min. Twelve milliliters of phosphate-buffered saline (PBS) was added; the suspension was centrifuged at 600 × *g* for 10 min, and the supernatant was discarded. This procedure was repeated twice. The pellet was frozen at −70°C until testing by nested PCR. To extend the DBS collection technique to a distant laboratory, an additional 30 samples were collected and processed as described for DBS and plasma at a laboratory in northern Thailand and shipped at room temperature (for DBS) or in ice packs (for plasma) by overnight bus to the serotyping laboratory. PCR was not performed on these specimens due to blood volume limitations.

A 5-mm hole punch was used to cut out filter disc DBS (discs were always completely saturated with blood with the 20-μl spot but not with the 5-μl spot), and individual discs were placed directly in 1 ml of specimen diluent (PBS [pH 7.4], 5% dry nonfat milk, and 0.1% Tween). Antibody was eluted overnight at 4°C. The eluate was then tested in parallel with 10 μl of plasma (assuming a 50% plasma yield from whole blood) from the same original specimen. An antigen-limiting V3-PEIA, as previously described (6), was modified in that each specimen was tested at a single dilution (1:100) against a range of peptide concentrations: 0.5, 0.05, and 0.005 μg/ml. The peptides used were V3-CM237 (CTRTPNNNTRKSIHLGP GKAWYTTGQIIGDIRQAH) and V3-CM242 (CTRPSNN TRTSITIGPGQVFYRTGDIIGDIRKAY), which have been previously shown to distinguish HIV-1 subtypes B and E in Thai subjects (1, 13). The cutoff value of the assay was deter-

* Corresponding author. Mailing address: Henry M. Jackson Foundation for the Advancement of Military Medicine, AFRIMS, 315/6 Rajvithi Road, Bangkok 10400, Thailand. Phone: 66-2-644-4888, ext. 1504. Fax: 66-2-644-4824. E-mail: mdesouza@hiv.hjf.org.

TABLE 1. Comparison of HIV-1 serotyping results with plasma and DBS from HIV-1-seropositive Thai subjects

Serotype from DBS[a]	No. of specimens with indicated serotype from plasma[b]				Total
	B	E	B/E	NR	
B	11 (92)	0	0	0	11
E	0	225 (99)	0	0	225
B/E	0	0	4 (80)	0	4
NR	1	1	1	14 (82)	17
Total	12	226	5	14	257

[a] DBS consisted of 20 μl of whole blood. NR, nonreactive.
[b] Values in parentheses represent percent concordance.

TABLE 2. Comparison of results of specimen subtyping based on genotyping of proviral DNA by PCR and serotyping with DBS (20 μl) and V3-PEIA

DBS HIV-1 V3 serotype	No. of specimens with indicated PCR HIV-1 genotype				Total
	B	E	Nontypeable	Nonamplifiable	
B	10	0	0	1	11
E	0	183	1	41	225
B/E	0	1	2	1	4
Nonreactive	2	11	0	4	17
Total	12	195	3	47	257

mined by the equation 2 × (mean of pooled HIV-1-seronegative samples + standard deviation). A fourfold or higher ratio of the optical density obtained at the same peptide concentration from one peptide relative to the other was used to assign the HIV-1 subtype. Eluted antibodies or plasma demonstrating reactivity to both peptides but at less than a fourfold differential were classified as dually reactive. Specimens having an optical density of equal to or less than the cutoff value for both peptides were classified as nonreactive.

Cell pellets were thawed and subjected to proteinase K digestion, and 0.5 to 1 μg of peripheral blood mononuclear cell (PBMC) DNA was assayed by nested PCR with primers from the *env* gp41 region, as previously described (1), and universal first-round primers and second-round primers capable of differentiating HIV-1 genotypes B and E. Amplification products were analyzed by electrophoresis on ethidium bromide-stained 1.5% agarose gels. Negative controls included PBMC DNA from HIV-1-seronegative donors amplified concurrently with the patient samples. Positive controls were PBMC DNA obtained from HIV-1-infected Thai subjects of known genotype, B or E. Samples which failed to amplify were scored as unamplifiable.

HIV-1 subtyping was successful with 243 (95%) plasma samples and 240 (93%) DBS samples, of the 257 specimens from HIV-1-infected subjects (Table 1). Most specimens were found to be HIV-1 subtype E. With plasma, 226 were typed as E, 12 as B, and 5 as dually reactive; with DBS, 225 were typed as E, 11 as B, and 4 as dually reactive. Three specimens were typeable by plasma but not DBS (one each B, E, and B/E). Both plasma and DBS samples from 14 subjects were nonreactive by peptide serology. All of the eight HIV-1-seronegative controls were nonreactive by both specimen collection methods. Use of a 5-μl volume of blood for DBS decreased the serotyping sensitivity of DBS by 12% (211 and 240 specimens were subtyped with 5 and 20 μl of DBS, respectively). Sensitivities for both serotypes were decreased (from 11 to 8 for B and from 225 to 199 for E), with the number of dual reactors unchanged. The additional 30 samples transported by overnight bus from the rural laboratory were obtained from 27 HIV-seropositive and 3 HIV-seronegative subjects. All samples from the seropositive subjects were subtyped as HIV subtype E by both the DBS and plasma methods, with the three HIV-seronegative samples being nonreactive for both DBS and plasma specimen collection.

PCR resulted in genotyping of 207 (81%) of the 257 specimens (Table 2). Twelve were typed as B, 195 were typed as E, 3 were amplified but could not be typed, and 47 failed to amplify. Subtype E was the predominant HIV-1 clade in the study population, a finding consistent with previous reports (11). Interestingly, PCR genotyping amplified both B and E products in two of the specimens which were serotyped as dual reactors by both DBS and plasma. This may represent genetic variants of HIV-1, specimen contamination, or subjects dually infected with both subtypes, as previously described (1). The rate of detection of HIV-1 by PCR was lower in this study than previously reported (6). The PCR method was unchanged, but sensitivity may have been affected by specimen preparation. Whole blood, rather than isolated PBMC, was used for DNA extraction in this study, and the porphyrin moiety of heme from contaminating erythrocytes has been shown to inhibit PCR (7). Alternatively, the use of EDTA tubes for venipuncture may have chelated magnesium necessary for PCR. This study assessed simplified diagnostic methods; hence, the nucleic acid extract was not subjected to any purification procedures. A direct comparison of HIV genotyping by nested PCR was made with EDTA-collected whole blood from 9 HIV-seropositive subjects. Two milliliters of blood was divided equally, and PBMC were prepared by either standard Ficoll-Hypaque separation (6) or the lysing method described in the present study. HIV-specific products were amplified with DNA obtained from eight of nine subjects (five were subtype E and three were subtype B) by the Ficoll technique and from five of nine subjects by the erythrocyte lysis procedure, implying that erythrocyte contamination was the major cause of PCR inhibition.

No samples were classified as one subtype by serology and another by PCR, although one dually amplified specimen by PCR was subtyped as HIV-1 clade E by peptide serotyping. With sera and plasma, it has been reported that HIV-1 serotype E shows high concordance with genetic subtyping (82 to 96%), while subtype B (Thailand) shows poor concordance (0 to 50%) with genetic typing, as assessed by three independent laboratories (2).

HIV-1 serotyping is an important epidemiological tool in Thailand, where two viral subtypes are currently circulating (11). These results show that DBS provides a reliable alternative to plasma for such testing. The current study used 20 μl of whole blood, but one blood drop corresponds to approximately 15 to 20 μl of whole blood, with a finger prick yielding approximately 10 to 20 drops (3). Our study demonstrates that DBS specimen collection could be used as a field-applicable epidemiological tool with more sophisticated genetic techniques used on dually reactive or nontypeable specimens from known HIV-positive subjects. The method has the great advantage of not requiring elaborate serum or plasma collection equipment, and DBS are easy to store and transport, providing an excellent option for HIV-1 serotyping in pediatric populations. Most importantly, the reduced use of glassware in DBS collection decreases the occupational exposure of health care workers to blood-borne pathogens.

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