Development and Application of a Broadly Sensitive Dried-Blood-Spot-Based Genotyping Assay for Global Surveillance of HIV-1 Drug Resistance[▽]

Chunfu Yang,^{1*} Amanda McNulty,¹ Karidia Diallo,¹ Jing Zhang,^{1,2} Boghuma Titanji,³ Sidibe Kassim,¹ Nellie Wadonda-Kabondo,⁴ John Aberle-Grasse,⁵ Tabitha Kibuka,⁶ Peter M. Ndumbe,³ Shanmugam Vedapuri,¹ Zhiyong Zhou,¹ Benson Chilima,⁴ and John N. Nkengasong¹

Division of Global AIDS, NCHHSTP, Centers for Disease Control and Prevention, Atlanta, Georgia¹; Institute of AIDS/HIV Control and Prevention, Shandong Center for Disease Control and Prevention, Shandong, China²; Center for the Study and Control of Communicable Diseases (CSCCD), University of Yaounde, Yaounde, Cameroon³; Community Health Sciences Unit, Malawi Ministry of Health,⁴ and CDC/GAP,⁵ Lilongwe, Malawi; and CDC/GAP, Dar es Salaam, Tanzania⁶

Received 16 March 2010/Returned for modification 5 May 2010/Accepted 14 July 2010

As antiretroviral therapy (ART) is scaled up in resource-limited countries, surveillance for HIV drug resistance (DR) is vital to ensure sustained effectiveness of first-line ART. We have developed and applied a broadly sensitive dried-blood-spot (DBS)-based genotyping assay for surveillance of HIV-1 DR in international settings. In 2005 and 2006, 171 DBS samples were collected under field conditions from newly diagnosed HIV-1-infected individuals from Malawi ($n = 58$), Tanzania ($n = 60$), and China ($n = 53$). In addition, 30 DBS and 40 plasma specimens collected from ART patients in China and Cameroon, respectively, were also tested. Of the 171 DBS analyzed at the protease and RT regions, 149 (87.1%) could be genotyped, including 49 (81.7%) from Tanzania, 47 (88.7%) from China, and 53 (91.4%) from Malawi. Among the 70 ART patient samples analyzed, 100% (30/30) of the Chinese DBS and 90% (36/40) of the Cameroonian plasma specimens were genotyped, including 8 samples with a viral load of <400 copies/ml. The results of phylogenetic analyses indicated that the subtype, circulating recombinant form (CRF), and unique recombinant form (URF) distribution was as follows: 73 strains were subtype C (34%), 37 were subtype B (17.2%), 24 each were CRF01_AE or CRF02_AG (11.2% each), 22 were subtype A1 (10.2%), and 9 were unclassifiable (UC) (4.2%). The remaining samples were minor strains comprised of 6 that were CRF07_BC (2.8%), 5 that were CRF10_CD (2.3%), 3 each that were URF_A1C and CRF08_BC (1.4%), 2 each that were G, URF_BC, and URF_D/UC (0.9%), and 1 each that were subtype F1, subtype F2, and URF_A1D (0.5%). Our results indicate that this broadly sensitive genotyping assay can be used to genotype DBS collected from areas with diverse HIV-1 group M subtypes and CRFs. Thus, the assay is likely to become a useful screening tool in the global resistance surveillance and monitoring of HIV-1 where multiple subtypes and CRFs are found.

Filter papers have been useful for the collection, storage, and testing of blood specimens in the diagnostic screening of metabolic and inherited disorders in newborn babies in the United States for many years (13, 14). More recently, many resource-limited countries have been using them for HIV-related molecular assays, including early infant diagnosis using Roche Amplicor HIV-1 DNA PCR testing (supported by the U.S. President's Emergency Plan for AIDS Relief [PEPFAR]) (31), real-time PCR-based HIV-1 diagnosis (22, 27) and viral load measurement (6, 17, 18, 20, 26), and HIV-1 drug resistance (DR) genotyping (5, 7, 8, 12, 16, 19, 23–25, 28, 30, 35–37).

Dried blood spots (DBS) offer several advantages over conventional plasma or serum for sample collection and storage. First, DBS circumvent the need for phlebotomy and reduce the risk of needle-stick-related HIV exposure when they are col-

lected using finger or heel stick. Second, they do not require cold-chain transportation of specimens from collection sites to the testing laboratories; thus, they can be transported using standard postal services, resulting in the reduction of cost for storage and transportation when dried and processed appropriately. Overall, DBS provide resource-limited countries with opportunities for sustainable laboratory services and surveillance programs for HIV diagnosis and DR genotyping. Very few studies have been conducted using DBS collected under real field conditions. Furthermore, the DR genotyping assays used were not evaluated for multiple HIV-1 subtypes and circulating recombinant forms (CRFs) (5, 16, 19, 23, 25, 37).

The aim of the current study was to determine the performance of a broadly sensitive genotyping assay for detecting multiple HIV-1 group M subtypes and CRFs that cocirculate in different countries, using DBS collected under field conditions.

MATERIALS AND METHODS

Study population. In 2005 and 2006, 171 DBS were collected under field conditions from individuals from Malawi ($n = 58$), Tanzania ($n = 60$), and China ($n = 53$) who were newly diagnosed with HIV-1 infections. In addition, specimens from antiretroviral therapy (ART) patients, including 30 DBS collected from Chinese patients in 2008 and 40 plasma specimens collected from HIV-1-

* Corresponding author. Mailing address: International Laboratory Branch, DGHA, NCHHSTP, CDC, Mail Stop A-11, 1600 Clifton Road, Atlanta, GA 30333. Phone: (404) 639-4975. Fax: (404) 718-1891. E-mail: cxy0@cdc.gov.

[▽] Published ahead of print on 21 July 2010.

infected Cameroon women in 2007, were also analyzed. To determine the specificity of the assay, 42 HIV-1-negative DBS specimens collected from women attending an antenatal clinic (ANC) in Tanzania were tested. All study protocols were approved by local and CDC institutional review boards. Anonymous genotyping was performed at the International Laboratory Branch, Division of Global AIDS, Centers for Disease Control and Prevention (CDC), Atlanta, GA. The detailed demographic and clinical information on the participants from Malawi, Tanzania, and China who were newly diagnosed with HIV-1 infection has been described elsewhere (19, 30, 36).

Dried-blood-spot collection, storage, and shipment. Dried-blood-spot specimens were made by spotting 100 μ l of whole blood collected into a Vacutainer containing K₃-EDTA onto Whatman 903 paper (Whatman, Inc., Piscataway, NJ) and drying overnight at ambient temperature. After drying, each DBS card was wrapped with a folded sheet of glassine paper, and 5 to 10 glassine paper-wrapped cards were packaged into a low-gas-permeable, zip closure plastic bag containing 8 to 10 desiccant bags and one humidity indicator (Fisher Scientific, Inc., Pittsburgh, PA). The DBS were stored at -70°C or -20°C before being shipped to Atlanta and were stored at -70°C in Atlanta until testing. The quality of the plasma samples received from Cameroon might have been compromised, since the samples were received under ambient-temperature conditions.

HIV serologic tests and viral load measurements. HIV serology tests for DBS specimens collected from newly diagnosed HIV-1-infected patients from Tanzania, Malawi, and China were repeated using the Genetic System HIV-1/HIV-2 peptide EIA kit (Bio-Rad Laboratories, Redmond, WA) and the Cambridge Biotech HIV-1 Western blot kit (Calypse Biomedical Corp., Rockville, MD). The HIV-1 viral loads in Cameroon plasma specimens were determined with an Amplicor HIV-1 monitor, version 1.5, which has a detection limit of 400 copies/ml (Roche Diagnostics, Indianapolis, IN). For samples collected from Chinese ART patients, the plasma viral loads were measured with the NASBA HIV-1 quantitation system, which has a detection limit of 50 copies/ml (Organon Teknika Baxtel, Netherlands), at Shandong Provincial CDC, Shandong, China. Due to the lack of available plasma specimens to pair with DBS collected from the newly HIV-1 diagnosed patients from Malawi, Tanzania, and China, the plasma viral loads in these patients were not determined.

Nucleic acid extraction. HIV-1 viral RNA was extracted from 140- μ l amounts of the Cameroon plasma samples using a QIAamp viral RNA kit, following the manufacturer's protocol (Qiagen, Valencia, CA). Total nucleic acids (TNA) were extracted from the DBS using a modified NucliSens silicon-based extraction procedure (bioMérieux, Inc., Durham, NC). In brief, one or two whole spots containing 100 μ l of whole blood were cut with scissors and added to 9 ml of NucliSens lysis buffer. Following a 2-hour incubation at room temperature under gentle rotation, the tubes were centrifuged at $250 \times g$ for 5 min and supernatant was transferred to a new 15-ml conical tube. TNA were extracted using NucliSens isolation reagents according to the manufacturer's procedure, resuspended in 50 μ l of elution buffer, and stored at -70°C before use.

Amplification of HIV-1 partial *pol* gene by RT-PCR and nested PCR. Primers were designed and modified (25) with the intention to amplify all HIV-1 group M subtypes and CRFs of the *pol* gene region associated with DR in the protease and reverse transcriptase (RT) regions of the HIV-1 genome. The outer primers for RT-PCR were Prt-F1 (forward, 5'-CCT CAA ATC ACT CTT TGG CAR CG, nucleotides [nt] 2253 to 2275 based on HXBII) and RT-R1 (reverse, 5'-ATC CCT GCA TAA ATC TGA CTT GC, nt 3370 to 3348), and the nested-PCR primers were Prt-F2 (forward, 5'-CTT TGG CAA CGA CCC CTY GTC WCA, nt 2265 to 2288) and RT-R2 (reverse, 5'-CTT CTG TAT GTC ATT GAC AGT CC, nt 3326 to 3304). For RT-PCR, 10 μ l of RNA or TNA extracts was used with primers Prt-F1 and RT-R1 (4 μ M each/50 μ l of RT-PCR mixture) and the SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase high fidelity, following the manufacturer's protocol (Invitrogen, Carlsbad, CA). For nested PCR, 4 to 8 μ l of each of the RT-PCR products was added to 100 μ l of the PCR mixture, containing 0.12 μ M (each) Prt-F2 and RT-R2 primers, 1 $\times$ GeneAmp gold buffer II, 2 mM MgCl₂, and 2.5 U of AmpliTaq gold LD DNA polymerase (Applied Biosystems, Foster City, CA). After initial denaturation at 94°C for 4 min, 40 cycles of PCR were performed in a GeneAmp 9700 thermocycler with PCR conditions of 94°C for 15 s, 55°C for 20 s, and 72°C for 2 min following an extension at 72°C for 10 min. Nested PCR products of 1,062 bp were confirmed by agarose gel electrophoresis, and purified products were used for sequencing.

Sensitivity and specificity of the *pol* genotyping primer pairs. The sensitivity of the genotyping primer set was determined using DBS and plasma specimens collected from ART patients with known plasma viral loads. To determine the specificity of the primer set, 42 HIV-negative DBS collected from pregnant women at a Tanzanian ANC were tested.

Sequence, drug resistance mutation, and phylogenetic analyses. Sequencing analysis of HIV-1 *pol* was performed using either an ABI 3100 or a 3730 DNA sequencer (Applied Biosystems, Foster City, CA). ChromasPro, version 1.42 (Technelysium Pty. Ltd., Tewantin, Australia), was used for editing sequences and producing consensus sequences. The protease and RT genotypes were interpreted using the Stanford Genotypic Resistance Interpretation Algorithm for specimens collected from ART patients and the Calibrated Population Resistance (CPR) tool for specimens collected from ART-naïve patients (<http://hivdb.stanford.edu/index.html>), as well as the WHO list for determination of transmitted HIV-1 DR (3).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA software, version 4 (32), and BioEdit (15) along with reference sequences from the HIV sequence database. After all gaps were trimmed, phylogenetic trees were constructed by the neighbor-joining method included in MEGA 4. The reliability of the tree topology was tested with 1,000 bootstrap analyses, and bootstrap values of $\geq 70\%$ were considered significant.

Nucleotide sequence accession numbers. The newly obtained sequences were submitted to GenBank with accession numbers HQ009559 to HQ009690.

RESULTS

Confirmation of HIV-1 infection and viral load measurement. The HIV status of the DBS specimens collected from patients newly diagnosed with HIV-1 was retested and confirmed. The median viral loads were 11,605 copies/ml (range, <400 to 367,875 copies/ml) for the 40 plasma samples from Cameroon and 4,620 copies/ml (range, 290 to 72,000 copies/ml) for DBS samples from China (Tables 1 and 2).

Determination of the sensitivity of the broadly sensitive genotyping assay. First, we evaluated the assay's sensitivity with 40 plasma specimens from ART patients from Cameroon, of which 36 (90%) were successfully genotyped. Of the 4 specimens that were not genotyped, two had viral loads of 1,341 and 1,391 copies/ml and the remaining two had viral load of 15,287 and 119,260 copies/ml. The inability to genotype these four specimens could likely be attributed to the compromised quality of the specimens (see Materials and Methods for details). However, it could also be a result of sequencing variations at the primer locations, since we were able to genotype two of the four specimens (2007693335 and 2007693340) (Table 1) with the Trugene HIV-1 genotyping system (Siemens Medical Solutions Diagnostics, Tarrytown, NY) after the broadly sensitive genotyping assay had failed. Sequence comparison analysis revealed that there are two nucleotide variations at positions 4 and 9 for the nested-PCR forward primer (Prt-F2) at the 3' side. However, it is worth noting that the assay was able to genotype all 6 specimens with a viral load of $<1,000$ copies/ml (Table 1).

Next, we measured the assay's sensitivity with DBS specimens collected from ART patients from Shandong Province, China. All 30 DBS specimens tested were genotyped, although some of the DBS specimens had viral loads below 1,000 copies/ml of plasma (Table 2).

Genotyping analyses using the Stanford Genotypic Resistance Interpretation Algorithm (<http://hivdb6.stanford.edu/asi/deployed/HIVdb.html>) indicated that 6 of the 38 Cameroon patients and 22 of the 30 Chinese ART patients had mutations associated with resistance to protease inhibitors (PI), nucleoside RT inhibitors (NRTI), and nonnucleoside RT inhibitors (NNRTI) (Table 3).

The phylogenetic analyses revealed that the predominant strains from Cameroon samples were CRF02_AG (24/36, 66.7%), followed by A1 and unclassifiable (UC) (4/36 of each,

TABLE 1. Amplification and genotyping efficiency of the broadly sensitive genotyping assay for plasma specimens collected from Cameroon ART patients, stratified by plasma viral load

Group, patient	ART regimen ^b	HIV-1 subtype or CRF	Viral load (copies/ml) ^a	RT-PCR result	Genotyping result
I			<1,000		
2007693347	sdNVP + 3TC + duovir	CRF02_AG	<400	+	+
2007693358	AZT + sdNVP + duovir	CRF02_AG	<400	+	+
2007693366	AZT + sdNVP	A1	<400	+	+
2007693371	sdNVP	CRF02_AG	<400	+	+
2007693360	AZT + sdNVP + duovir	CRF02_AG	869	+	+
2007693341	AZT + sdNVP	CRF02_AG	883	+	+
II			1,000–10,000		
2007693337	sdNVP + 3TC + duovir	CRF02_AG	1,111	+	+
2007693364	AZT + sdNVP	NA ^c	1,341	–	–
2007693335	sdNVP + duovir	CRF02_AG ^d	1,391	–	–
2007693355	sdNVP + 3TC + duovir	F2	1,536	+	+
2007693349	sdNVP + 3TC + duovir	CRF02_AG	2,320	+	+
2007693367	AZT + sdNVP	CRF02_AG	2,630	+	+
2007693351	sdNVP	F1	3,226	+	+
2007693362	sdNVP + 3TC + duovir	CRF02_AG	3,588	+	+
2007693353	AZT + sdNVP	CRF02_AG	5,395	+	+
2007693345	AZT + sdNVP	G	6,900	+	+
2007693368	AZT + sdNVP	CRF02_AG	7,310	+	+
2007693356	AZT + sdNVP	CRF02_AG	8,732	+	+
III			>10,000–100,000		
2007693339	AZT + sdNVP	CRF02_AG	10,883	+	+
2007693352	AZT + sdNVP	G	11,324	+	+
2007693369	AZT + sdNVP	UC ^e	11,885	+	+
2007693370	sdNVP	CRF02_AG	11,885	+	+
2007693373	sdNVP + 3TC + duovir	NA	15,287	–	–
2007693342	sdNVP + 3TC + duovir	CRF02_AG	16,015	+	+
2007693348	AZT + sdNVP	CRF02_AG	21,942	+	+
2007693343	sdNVP + 3TC + duovir	A1	24,226	+	+
2007693336	AZT + sdNVP	CRF02_AG	25,981	+	+
2007693359	sdNVP + 3TC + duovir	A1	27,229	+	+
2007693361	sdNVP + 3TC + duovir	CRF02_AG	27,595	+	+
2007693354	AZT + sdNVP	UC	28,050	+	+
2007693363	AZT + sdNVP	CRF02_AG	45,403	+	+
2007693334	sdNVP + 3TC + duovir	CRF02_AG	53,568	+	+
2007693372	sdNVP	CRF02_AG	54,471	+	+
2007693338	sdNVP + 3TC + duovir	CRF02_AG	91,379	+	+
IV			>100,000		
2007693340	sdNVP + 3TC + duovir	CRF02_AG ^d	119,260	–	–
2007693346	AZT + sdNVP	UC	119,769	+	+
2007693344	sdNVP + 3TC + duovir	CRF02_AG	182,126	+	+
2007693350	sdNVP + 3TC + duovir	A1	293,634	+	+
2007693357	AZT + sdNVP	UC	301,649	+	+
2007693365	sdNVP	CRF02_AG	367,875	+	+

^a The detection limit of the Roche Amplicor HIV-1 viral load assay, version 1.5, was 400 copies/ml of plasma.^b sdNVP, single-dose nevirapine; 3TC, lamivudine; duovir, generic combivir (3TC and zidovudine); AZT, zidovudine.^c NA, not available.^d Genotyping was performed with the Trugene HIV-1 genotyping system after genotyping with the broadly sensitive genotyping assay had failed.^e UC, unclassifiable.

11.1%), G (2/36, 5.6%), and F1 and F2 (1/36 of each, 2.8%). The subtype distributions from DBS specimens collected from China were 90% (27/30) B and 10% (3/30) CRF01_AE.

Specificity of the newly developed genotyping assay. To evaluate the specificity of the genotyping assay, we tested 42 HIV-1-negative DBS collected from women attending an ANC in Tanzania. These specimens were confirmed to be HIV-1 negative with serologic tests at CDC, Atlanta, GA. Genotyping analysis revealed that they were all negative by RT-PCR using the broadly sensitive genotyping assay.

Application of the broadly sensitive genotyping assay in the surveillance of transmitted HIV-1 drug resistance in resource-limited countries. Surveillance of transmitted DR in resource-limited settings has become an urgent task with the scaling up of ART in these countries. The initial characterization of the new assay has revealed that this assay might be suitable for populations infected with non-B HIV-1 subtypes and CRFs. We received 171 DBS specimens from 3 countries where transmitted-DR surveys (19, 30, 36) were conducted using the WHO HIV DR Threshold Survey (HIVDR-TS) protocol (4).

TABLE 2. Genotyping sensitivity of the broadly sensitive genotyping assay for DBS specimens collected from ART patients from Shandong Province, China

Sample	Identification no.	ART regimen ^b	HIV-1 subtype or CRF	Viral load (copies/ml) ^a	RT-PCR result	Genotyping result
1	CNsd5192	d4T + 3TC + EFV	B	290	+	+
2	CNsd0372	3TC + d4T + NVP	B	317	+	+
3	CNsd0705	d4T + 3TC + NVP	B	340	+	+
4	CNsd5210	d4T + 3TC + NVP	B	390	+	+
5	CNsd0409	3TC + d4T + NVP	B	570	+	+
6	CNsd0576	ddI + NVP + d4T	B	730	+	+
7	CNsd0424	AZT + ddI + NVP	B	1,000	+	+
8	CNsd0320	AZT + ddI + NVP	B	1,300	+	+
9	CNsd0227	AZT + ddI + NVP	B	1,400	+	+
10	CNsd0402	AZT + ddI + NVP	B	1,600	+	+
11	CNsd5160	ddI + d4T + NVP	B	1,600	+	+
12	CNsd5162	d4T + ddI + NVP	B	2,900	+	+
13	CNsd5141	3TC + d4T + NVP	B	3,100	+	+
14	CNsd0325	AZT + ddI + NVP	B	3,200	+	+
15	CNsd5164	AZT + ddI + NVP	B	4,600	+	+
16	CNsd5152	AZT + 3TC + NVP	B	4,700	+	+
17	CNsd0322	AZT + ddI + NVP	B	4,900	+	+
18	CNsd0662	3TC + d4T + NVP	CRF01_AE	6,000	+	+
19	CNsd0312	d4T + 3TC + NVP	B	9,700	+	+
20	CNsd0417	AZT + ddI + NVP	B	9,800	+	+
21	CNsd7122	3TC + d4T + NVP	B	10,000	+	+
22	CNsd0430	AZT + ddI + NVP	B	12,000	+	+
23	CNsd5144	AZT + ddI + NVP	B	12,000	+	+
24	CNsd0414	AZT + ddI + NVP	B	16,000	+	+
25	CNsd5160	d4T + ddI + NVP/AZT + 3TC + NVP	B	23,000	+	+
26	CNsd0525	3TC + d4T + NVP	CRF01_AE	40,000	+	+
27	CNsd0216	AZT + ddI + EFV	B	54,000	+	+
28	CNsd0334	AZT + ddI + NVP/3TC + d4T + EFV	B	61,000	+	+
29	CNsd0350	AZT + ddI + NVP	B	69,000	+	+
30	CNsd0423	AZT + ddI + NVP	CRF01_AE	72,000	+	+

^a Viral load was measured with the NASBA HIV-1 quantitation system, which has a detection limit of 50 copies/ml of plasma.

^b d4T, stavudine; 3TC, lamivudine; EFV, efavirenz; ddI, didanosine; AZT, zidovudine; NVP, nevirapine.

Of the 149 (149/171, 87.1%) successfully genotyped DBS samples, 49 (49/60, 81.7%) were from Tanzania, 47 (47/53, 88.7%) from China, and 53 (53/58, 91.4%) from Malawi. Genotyping analysis revealed that all the specimens had no transmitted-DR mutations, except that one specimen from China carried the K101E mutation to NNRTI based on the WHO list (3) and CPR criteria. Phylogenetic analyses revealed that the predominant subtype was C (49.0%), followed by CRF01_AE (14.1%), A1 (12.1%), B (6.7%), CRF07_BC (4.0%), CRF10_CD (3.4%), and UC (3.4%). The remaining strains were unique recombinant form A1C (URF_A1C), CRF08_BC, URF_D/UC, URF_BC, and URF_A1D, at $\leq 2\%$ prevalence (Table 4). It is noticeable that all the HIV-1 strains from Malawi belonged to subtype C, while the strains from China and Tanzania had very diverse subtypes, CRFs, and URFs.

DISCUSSION

In this study, we report the development of a broadly sensitive RT-PCR-based genotyping assay for surveillance and monitoring of HIV-1 DR in the *pol* gene region. By applying this assay, we genotyped DBS from 3 countries for transmitted HIV-1 DR. This study confirms and extends previous reports that DBS are a useful sample collection device for HIV-1 DR genotyping analysis (5, 7, 8, 12, 16, 19, 23–25, 28, 30, 35–37).

However, the current study provides additional support for the use of DBS for HIV DR genotyping in several ways. (i) Samples were collected under routine clinical conditions and transported to CDC Atlanta for genotyping. (ii) We genotyped multiple HIV-1 group M subtypes and CRFs in samples from China, Cameroon, Malawi, and Tanzania, reported in this study, and also from many other countries, including Botswana, Honduras, Ethiopia, Kenya, Nigeria, Thailand, and Zambia (data not shown). Together, these results indicated that this genotyping assay is broadly sensitive for all the major HIV-1 subtypes and CRFs. (iii) The assay is one-step RT-PCR-based and covers both the protease (amino acids [aa] 13 to 99) and RT (aa 1 to 251) regions, which results in the reduction of genotyping cost and the possibility of laboratory-related cross-contaminations because fewer hands-on manipulations are needed. Compared to commercially available genotyping assays, our assay reduces the cost for genotyping reagents by almost 50%. (iv) Lastly, the assay can be successful in genotyping at least 90% of samples collected under resource-limited field conditions and with a viral load of ≥ 400 copies/ml of plasma. However, the current 5' nested-PCR primer (Prt-F2) for the protease region starting at codon 13 is a few amino acids shorter than those in commonly used genotyping assays, such as the Trugene HIV-1 genotyping system (Siemens

TABLE 3. Resistance-associated mutations in protease and RT from plasma samples collected from Cameroon women participating in the Preventing Mother-to-Child Transmission Program and DBS samples collected from ART patients from China

Sample	Origin	Specimen type	HIV-1 subtype or CRF	Resistance mutation(s) to PI or RTI	
				Protease (aa 13–99)	Reverse transcriptase (aa 1–251)
2007693355	Cameroon	Plasma	F2	None	V118I
2007693362	Cameroon	Plasma	CRF02_AG	None	T215A
2007693353	Cameroon	Plasma	CRF02_AG	None	K103N
2007693339	Cameroon	Plasma	CRF02_AG	E35EG	None
2007693340	Cameroon	Plasma	CRF02_AG	E35EG	None
2007693368	Cameroon	Plasma	CRF02_AG	None	V106A
CNsd 5192	China	DBS	B	A71V	None
CNsd 0372	China	DBS	B	A71V	Y188FLY
CNsd 0705	China	DBS	B	None	V106I
CNsd 5210	China	DBS	B	A71V	None
CNsd 0409	China	DBS	B	A71V	None
CNsd 0576	China	DBS	B	A71V	None
CNsd 0227	China	DBS	B	None	M41L, V108I, Y181C, T215Y,
CNsd 0402	China	DBS	B	None	A62V, V75I, F77L, F116Y, Q151 M, G190A
CNsd 5160	China	DBS	B	A71T	K101E, E138T, Y181C
CNsd 5162	China	DBS	B	A71V	K65R, V108I, Y181C
CNsd 5141	China	DBS	B	None	K101HKNQ, V106 M, V108I, M184V, T215FS, F227L
CNsd 0325	China	DBS	B	None	K103N, Y181C, L210W, T215Y,
CNsd 5164	China	DBS	B	A71AT	M41L, V106A, L210W, T215Y, F227L
CNsd 5152	China	DBS	B	None	V179D
CNsd 0322	China	DBS	B	A71T	T215A
CNsd 0312	China	DBS	B	None	K103N, M184V, T215F,
CNsd 0430	China	DBS	B	None	K103N, M184V, T215F,
CNsd 5144	China	DBS	B	A71V	A62AV, V75IV, F116FY, Q151KLMQ, V179D, Y181C, H221HY
CNsd 5160	China	DBS	B	A71T	K70R, K101E, E138T, Y181C
CNsd 0334	China	DBS	B	A71T	M41L, D67DN, K70KR, V106A, L210LW, T215Y, H221HY, F227L
CNsd 0350	China	DBS	B	A71T	None
CNsd 0423	China	DBS	CRF01_AE	None	V179T

Medical Solutions Diagnostics, Tarrytown, NY) and the Viroseq HIV-1 genotyping system (Celera Diagnostics, Alameda, CA), and does not cover all the amino acid positions associated with DR against protease inhibitors. Currently, it will not affect the genotyping results, as many countries are only using the first-line regimens recommended by WHO (34), which do not include protease inhibitors. We are currently evaluating a new primer set to overcome this issue.

The unusually high viral diversity of HIV-1 poses problems historically for HIV diagnosis using nucleic acid-based tests (1, 9, 21). The two commercially available genotyping assays, the Viroseq HIV-1 genotyping system (Celera, Alameda CA) and the TruGene HIV-1 genotyping system (Siemens Medical Solutions Diagnostics, Tarrytown, NY), were designed and approved by the FDA for HIV-1 subtype B only. Although stud-

ies have indicated that these assays can be used for other HIV-1 group M subtypes, the reported sensitivities vary (2, 10, 11, 29, 33). The assay we report here and the one reported recently by Buckton et al. (7) are the only assays that have directly addressed subtype-genotyping-efficiency issues. In the analysis of Buckton et al., using plasmid clones representing major HIV-1 group M subtypes, they found that the genotyping assay was less efficacious for subtypes A2, CRF01_AE, CRF02_AG, F, and H in the RT region and for CRF02_AG, F, and H in the protease region, respectively. We have genotyped DBS and plasma specimens from more countries than reported here using our broadly sensitive genotyping assay. We found that the genotyping efficacies ranged from 90% to 100% for plasma samples (data not shown) and from 82% to 100% for DBS samples represent-

TABLE 4. Phylogenetic classification of DBS specimens collected from newly diagnosed HIV-1-infected persons from China, Malawi, and Tanzania

Origin	No. of DBS specimens	No. (%) with phylogenetic classification											
		C	CRF01_AE	A1	B	CRF07_BC	CRF10_CD	UC	CRF08_BC	URF_A1/C	D/UC	URF_BC	URF_A1C
Malawi	53	53	0	0	0	0	0	0	0	0	0	0	0
China	47	3	21	0	10	6	0	1	3	1	0	2	0
Tanzania	49	17	0	18	0	0	5	4	0	2	2	0	1
Total	149	73 (49.0)	21 (14.1)	18 (12.1)	10 (6.7)	6 (4.0)	5 (3.4)	5 (3.4)	3 (2.0)	3 (2.0)	2 (1.3)	2 (1.3)	1 (0.7)

ing HIV-1 group M subtypes A1, A2, B, C, D, F1, F2, and G, circulating recombinant forms CRF01_AE, CRF02_AG, CRF07_BC, CRF08_BC, and CRF10_CD, and many unique recombinant forms (Tables 1, 2, and 4).

In summary, we have developed a broadly sensitive genotyping assay that is restricted to the *pol* gene region. Using this assay, we were able to genotype diverse HIV-1 group M viral strains from DBS samples collected under field conditions in several countries where multiple subtypes and CRFs are co-circulating. The assay is likely to become a useful tool for HIV DR surveillance in many developing countries.

ACKNOWLEDGMENTS

Jing Zhang was a recipient of an 2008-2009 International Emerging Infectious Diseases (IEID) fellowship sponsored by the Association of Public Health Laboratories (APHL) and the CDC.

We express our sincere appreciation and thanks to all the field staff and participants for their support for the study and to the staff of the HIV Incidence and Serology Team of the International Laboratory Branch for performing HIV-1 confirmation tests at the CDC.

The use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

REFERENCES

- Alaesus, A., K. Lidman, A. Sonnerborg, and J. Albert. 1997. Subtype-specific problems with quantification of plasma HIV-1 RNA. *AIDS* 11: 859-865.
- Beddows, S., S. Galpin, S. H. Kazmi, A. Ashraf, A. Johargy, A. J. Frater, N. White, R. Braganza, J. Clarke, M. McClure, and J. N. Weber. 2003. Performance of two commercially available sequence-based HIV-1 genotyping systems for the detection of drug resistance against HIV type 1 group M subtypes. *J. Med. Virol.* 70:337-342.
- Bennett, D. E., R. J. Camacho, D. Otelea, D. R. Kuritzkes, H. Fleury, M. Kiuchi, W. Heneine, R. Kantor, M. R. Jordan, J. M. Schapiro, A. M. Vandamme, P. Sandstrom, C. A. Boucher, D. van de Vijver, S. Y. Rhee, T. F. Liu, D. Pillay, and R. W. Shafer. 2009. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS ONE* 4:e4724.
- Bennett, D. E., M. Myatt, S. Bertagnolio, D. Sutherland, and C. F. Gilks. 2008. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. *Antivir. Ther.* 13(Suppl. 2):25-36.
- Bertagnolio, S., L. Soto-Ramirez, R. Pilon, R. Rodriguez, M. Viveros, L. Fuentes, P. R. Harrigan, T. Mo, D. Sutherland, and P. Sandstrom. 2007. HIV-1 drug resistance surveillance using dried whole blood spots. *Antivir. Ther.* 12:107-113.
- Brambilla, D., C. Jennings, G. Aldrovandi, J. Bremer, A. M. Comeau, S. A. Cassol, R. Dickover, J. B. Jackson, J. Pitt, J. L. Sullivan, A. Butcher, L. Grosso, P. Reichelderfer, and S. A. Fiscus. 2003. Multicenter evaluation of use of dried blood and plasma spot specimens in quantitative assays for human immunodeficiency virus RNA: measurement, precision, and RNA stability. *J. Clin. Microbiol.* 41:1888-1893.
- Buckton, A. J., S. L. Bissett, R. E. Myers, S. Beddows, S. Edwards, P. A. Cane, and D. Pillay. 2008. Development and optimization of an internally controlled dried blood spot assay for surveillance of human immunodeficiency virus type-1 drug resistance. *J. Antimicrob. Chemother.* 62:1191-1198.
- Cassol, S. A., S. Read, B. G. Weniger, P. Gomez, N. Lapointe, C. Y. Ou, and P. G. Babu. 1996. Dried blood spots collected on filter paper: an international resource for the diagnosis and genetic characterization of human immunodeficiency virus type-1. *Mem. Inst. Oswaldo Cruz* 91:351-358.
- Eberle, J., I. Loussert-Ajaka, S. Brust, L. Zekeng, P. H. Hauser, L. Kaptue, S. Knapp, F. Damond, S. Saragosti, F. Simon, and L. G. Gurtler. 1997. Diversity of the immunodominant epitope of gp41 of HIV-1 subtype O and its validity for antibody detection. *J. Virol. Methods* 67:85-91.
- Eshleman, S. H., G. Becker-Pergola, M. Deseyve, L. A. Guay, M. Mracna, T. Fleming, S. Cunningham, P. Musoke, F. Mmiro, and J. B. Jackson. 2001. Impact of human immunodeficiency virus type 1 (HIV-1) subtype on women receiving single-dose nevirapine prophylaxis to prevent HIV-1 vertical transmission (HIV network for prevention trials 012 study). *J. Infect. Dis.* 184: 914-917.
- Fontaine, E., C. Riva, M. Peeters, J. C. Schmit, E. Delaporte, K. Van Laethem, K. Van Vaerenbergh, J. Snoeck, E. Van Wijngaerden, E. De Clercq, M. Van Ranst, and A. M. Vandamme. 2001. Evaluation of two commercial kits for the detection of genotypic drug resistance on a panel of HIV type 1 subtypes A through J. *J. Acquir. Immune Defic. Syndr.* 28:254-258.
- Garcia-Lerma, J. G., A. McNulty, C. Jennings, D. Huang, W. Heneine, and J. W. Bremer. 2009. Rapid decline in the efficiency of HIV drug resistance genotyping from dried blood spots (DBS) and dried plasma spots (DPS) stored at 37 degrees C and high humidity. *J. Antimicrob. Chemother.* 64: 33-36.
- Garrick, M. D., P. Dembure, and R. Guthrie. 1973. Sick-cell anemia and other hemoglobinopathies. Procedures and strategy for screening employing spots of blood on filter paper as specimens. *N. Engl. J. Med.* 288:1265-1268.
- Garrick, M. D., A. P. Orfanos, L. Rogers, E. W. Naylor, and R. Guthrie. 1981. A simple screening test for reduced glutathione in filter paper spots of blood. *J. Pediatr.* 98:265-267.
- Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 96/98/NT. *Nucleic Acids Symp. Ser.* 41:95-98.
- Hallack, R., L. E. Doherty, J. A. Wethers, and M. M. Parker. 2008. Evaluation of dried blood spot specimens for HIV-1 drug-resistance testing using the Trugene HIV-1 genotyping assay. *J. Clin. Virol.* 41:283-287.
- Hamers, R. L., P. W. Smit, W. Stevens, R. Schuurman, and T. F. Rinke de Wit. 2009. Dried fluid spots for HIV type-1 viral load and resistance genotyping: a systematic review. *Antivir. Ther.* 14:619-629.
- Johannessen, A., C. Garrido, N. Zahonero, L. Sandvik, E. Naman, S. L. Kivuyo, M. J. Kasubi, S. G. Gundersen, J. N. Bruun, and C. de Mendoza. 2009. Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania. *Clin. Infect. Dis.* 49:976-981.
- Kamoto, K., J. Aberle-Grasse, and Malawi HIV Drug Resistance Task Force. 2008. Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. *Antivir. Ther.* 13(Suppl. 2):83-87.
- Kane, C. T., H. D. Ndiaye, S. Diallo, I. Ndiaye, A. S. Wade, P. A. Diaw, A. Gaye-Diallo, and S. Mboup. 2008. Quantitation of HIV-1 RNA in dried blood spots by the real-time NucliSENS EasyQ HIV-1 assay in Senegal. *J. Virol. Methods* 148:291-295.
- Loussert-Ajaka, I., M. L. Chaix, B. Korber, F. Letourneur, E. Gomas, E. Allen, T. D. Ly, F. Brun-Vezinet, F. Simon, and S. Saragosti. 1995. Variability of human immunodeficiency virus type 1 group O strains isolated from Cameroonian patients living in France. *J. Virol.* 69:5640-5649.
- Luo, W., H. Yang, K. Rathbun, C. P. Pau, and C. Y. Ou. 2005. Detection of human immunodeficiency virus type 1 DNA in dried blood spots by a duplex real-time PCR assay. *J. Clin. Microbiol.* 43:1851-1857.
- Masciotra, S., C. Garrido, A. S. Youngpairaj, A. McNulty, N. Zahonero, A. Corral, W. Heneine, C. de Mendoza, and J. G. Garcia-Lerma. 2007. High concordance between HIV-1 drug resistance genotypes generated from plasma and dried blood spots in antiretroviral-experienced patients. *AIDS* 21:2503-2511.
- McNulty, A., K. Diallo, J. Zhang, B. Titanji, S. Kassim, D. Bennett, J. Abert-Grasse, T. Kibuka, P. M. Nduibe, J. N. Nkengasong, and C. Yang. 2008. Development and application of a broadly sensitive genotyping assay for surveillance of HIV-1 drug resistance in PEPFAR countries. *Antivir. Ther.* 13:A117.
- McNulty, A., C. Jennings, D. Bennett, J. Fitzgibbon, J. W. Bremer, M. Ussery, M. L. Kalish, W. Heneine, and J. G. Garcia-Lerma. 2007. Evaluation of dried blood spots for human immunodeficiency virus type 1 drug resistance testing. *J. Clin. Microbiol.* 45:517-521.
- Monleau, M., C. Montavon, C. Laurent, M. Segondy, B. Montes, E. Delaporte, F. Boillot, and M. Peeters. 2009. Evaluation of different RNA extraction methods and storage conditions of dried plasma or blood spots for human immunodeficiency virus type 1 RNA quantification and PCR amplification for drug resistance testing. *J. Clin. Microbiol.* 47:1107-1118.
- Ou, C. Y., H. Yang, S. Balinandi, S. Sawadogo, V. Shanmugam, P. M. Tih, C. de-Tourne, S. Tancho, L. K. Ya, M. Bulterys, R. Downing, and J. N. Nkengasong. 2007. Identification of HIV-1 infected infants and young children using real-time RT-PCR and dried blood spots from Uganda and Cameroon. *J. Virol. Methods* 144:109-114.
- Plantier, J. C., R. Dachraoui, V. Leme, M. Gueudin, F. Borsa-Lebas, F. Clanton, and F. Simon. 2005. HIV-1 resistance genotyping on dried serum spots. *AIDS* 19:391-397.
- Ribas, S. G., L. Heyndrickx, P. Ondoa, and K. Fransen. 2006. Performance evaluation of the two protease sequencing primers of the Trugene HIV-1 genotyping kit. *J. Virol. Methods* 135:137-142.
- Somi, G. R., T. Kibuka, K. Diallo, T. Tuhuma, D. E. Bennett, C. Yang, C. Kagoma, E. F. Lyamuya, R. O. Swai, and S. Kassim. 2008. Surveillance of transmitted HIV drug resistance among women attending antenatal clinics in Dar es Salaam, Tanzania. *Antivir. Ther.* 13(Suppl. 2):77-82.
- Stevens, W., G. Sherman, R. Downing, L. M. Parsons, C. Y. Ou, S. Crowley, G. M. Gershy-Damet, K. Fransen, M. Bulterys, L. Lu, J. Homsy, T. Finkbeiner, and J. N. Nkengasong. 2008. Role of the laboratory in ensuring global access to ARV treatment for HIV-infected children: consensus state-

- ment on the performance of laboratory assays for early infant diagnosis. *Open AIDS J.* **2**:17–25.
32. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–1599.
33. **Tong, C. Y., J. Mullen, R. Kulasegaram, A. De Ruiter, S. O'Shea, and I. L. Chrystie.** 2005. Genotyping of B and non-B subtypes of human immunodeficiency virus type 1. *J. Clin. Microbiol.* **43**:4623–4627.
34. **World Health Organization.** 2004. Scaling up antiretroviral therapy in resource-limited settings: treatment guidelines for a public health approach, 2003 revision. World Health Organization, Geneva, Switzerland.
35. **Youngpairoj, A. S., S. Masciotra, C. Garrido, N. Zahonero, C. de Mendoza, and J. G. Garcia-Lerma.** 2008. HIV-1 drug resistance genotyping from dried blood spots stored for 1 year at 4 degrees C. *J. Antimicrob. Chemother.* **61**:1217–1220.
36. **Zhang, J., D. Kang, J. Fu, X. Sun, B. Lin, Z. Bi, J. N. Nkengasong, and C. Yang.** 2010. Surveillance of transmitted HIV type 1 drug resistance in newly diagnosed HIV type 1-infected patients in Shandong Province, China. *AIDS Res. Hum. Retroviruses* **26**:99–103.
37. **Ziemniak, C., A. George-Agwu, W. J. Moss, S. C. Ray, and D. Persaud.** 2006. A sensitive genotyping assay for detection of drug resistance mutations in reverse transcriptase of HIV-1 subtypes B and C in samples stored as dried blood spots or frozen RNA extracts. *J. Virol. Methods* **136**:238–247.