AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

Simultaneous Detection of Major Drug Resistance Mutations of HIV-1 Subtype B Viruses from Dried Blood Spot Specimens by Multiplex Allele-Specific Assay

Guoqing Zhang,[a] Fangping Cai,[b] Ivette Lorenzana de Rivera,[c] Zhiyong Zhou,[a] Jing Zhang,[a] John Nkengasong,[a] Feng Gao,[b] Chunfu Yang[a]

International Laboratory Branch, Division of Global HIV/AIDS, Center for Global Health, CDC, Atlanta, Georgia, USA[a]; Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA[b]; Microbiology Department, National Autonomous University of Honduras, Tegucigalpa, Honduras[c]

A multiplex allele-specific (MAS) assay has been developed for the detection of HIV-1 subtype C drug resistance mutations (DRMs). We have optimized the MAS assay to determine subtype B DRMs in dried blood spots (DBS) collected from patients on antiretroviral therapy. The new assay accurately detected DRMs, including low-abundance mutations that were often missed by Sanger sequencing.

There were approximately 36.9 million people worldwide living with HIV at the end of 2014 (1). Despite significant advances in antiretroviral therapy (ART), the acquisition and transmission of HIV drug resistance (HIVDR) pose challenges to the continuous success of ART programs (2). The World Health Organization recommends that HIV treatment scale-up should always be accompanied by a robust assessment of drug resistance emergence and transmission (3).

Applying suspension array technology (4), we developed a multiplex allele-specific (MAS) assay that simultaneously detects major HIV drug resistance mutations (DRMs) at 20 loci in subtype C viruses (5). Because of significant genetic variation between different HIV-1 subtypes, not all allele-specific primer extension (ASPE) primers designed on the basis of subtype C sequences will work for other subtypes (5, 6). Since subtype B is dominant in many countries in the Americas, Europe, Asia, and Africa (7), we modified the original subtype C MAS assay to detect DRMs in subtype B viruses and evaluated the performance of the MAS assay with dried blood spots (DBS) collected from patients on ART in this study.

(These data were presented in part at the International Workshop on HIV & Hepatitis Virus Drug Resistance and Curative, 5 to 9 June 2012, Sitges, Spain.)

We modified the 45 subtype C ASPE primers targeting the DRMs at 20 loci that are associated with resistance to commonly used antiretrovirals. These include major nucleoside reverse transcriptase inhibitor (NRTI) DRMs at eight loci (M41L, K65R, K70R, L74V, Y115F, Q151M, M184V, and K219Q/E), major non-NRTI DRMs at seven loci (L100I, K101P/E, K103N/R, V106A/M, Y181C, Y188L, and G190A), and major protease inhibitor DRMs at five loci (V32I, I47A/V, L76V, I84V, and L90M). The lengths of the modified primers were between 24 and 39 nucleotides, and the melting temperatures (T_ms) were around 60°C. For a summary of all 45 modified ASPE primer sequences, see Table S1 in the supplemental material.

After the assay was optimized for the detection of all of the DRMs with plasmids for several factors affecting specificity and signal output, such as Mg^{2+} concentration, cycling parameters, annealing temperature, and ASPE primer concentrations (5), DBS samples collected from 69 subtype B-infected patients undergoing ART were used to evaluate the modified MAS assay (8). This study was approved by the ethics committee at the National Autonomous University of Honduras. The Center for Global Health at CDC approved the study protocol as a research activity involving unlinked or anonymous data or specimens collected for another purpose. Total nucleic acid extracted from these DBS samples was subjected to reverse transcription and nested PCR amplification with a validated HIVDR genotyping assay (9, 10). The nested PCR products were then analyzed by the MAS assay, and the results were compared to Sanger sequencing results (9). To verify the additional low-abundance alleles detected by the MAS assay, selected DBS samples with low-abundance alleles were analyzed by next-generation sequencing with Roche 454 GS-FLX Titanium sequencing kit XLR70 (Roche Applied Science, Indianapolis, IN, USA) (5).

The agreement between the two assays for the detection of resistance at the patient and DRM levels was assessed by kappa analysis. Statistical calculations were performed with SPSS 21.0 (IBM SPSS Inc., Armonk, NY).

Of the 1,380 loci analyzed in the 69 DBS samples (20 loci per specimen), 1,340 (97.10%; 95% confidence interval [CI], 96.08 to 97.86%) were successfully genotyped; the remaining 40 loci (2.90%) could not be determined because of negative reactions for both wild-type (WT) and mutant (Mut) alleles. Of the 1,340 genotypes detected by both the MAS assay and Sanger sequencing, the results obtained by the two methods with 1,330 (99.25%; 95% CI, 98.63 to 99.59%) were identical and those obtained with 10 were discordant. Of the 10 discordant genotypes, 8 alleles were

Received 22 October 2015 **Accepted** 2 November 2015

Accepted manuscript posted online 11 November 2015

Citation Zhang G, Cai F, de Rivera IL, Zhou Z, Zhang J, Nkengasong J, Gao F, Yang C. 2016. Simultaneous detection of major drug resistance mutations of HIV-1 subtype B viruses from dried blood spot specimens by multiplex allele-specific assay. J Clin Microbiol 54:220–222. doi:10.1128/JCM.02833-15.

Editor: Y.-W. Tang

Address correspondence to Chunfu Yang, cyang1@cdc.gov, or Feng Gao, fgao@duke.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02833-15.

TABLE 1 Comparison of HIV drug resistance determined by Sanger sequencing and the MAS assay

MAS	Sanger sequencing			Kappa	*P*
	Positive	Negative	Total		
At specimen level					
Positive	37	0	37	1.00	<0.001
Negative	0	32	32		
Total	37	32	69		
At mutation level[a]					
Positive	85	6	91	0.96	0.02
Negative	1	1,248	1,249		
Total	86	1,254	1,340		

[a] Forty mutation loci that yielded indeterminate results by the MAS assay were not included.

considered mixtures of WT and Mut genotypes by the MAS assay while they were classified as nonmixtures (5 WT and 3 Mut genotypes) by Sanger sequencing (see Table S2 in the supplemental material). One allele identified as a mixture by Sanger sequencing was identified as a WT allele by the MAS assay, and another one determined as a WT allele by Sanger sequencing was classified as a Mut allele by the MAS assay (see Table S2). Five specimens with low-abundance alleles were amplified and subjected to next-generation sequencing. All low-abundance alleles (K103KN in specimen HN103, K65KR in HN15, Y115YF in HN32, K101KE in HN101, and V106 in HN106) detected by MAS were verified by ultradeep sequencing. The results showed that the proportions of low-abundance Mut alleles missed by Sanger sequencing were 1.39 to 22.11%.

Both the MAS assay and Sanger sequencing detected DRMs in 37 specimens and no DRMs in 32 specimens. Thus, there was perfect agreement between the two methods in DR prediction at the patient level (kappa = 1.00; $P < 0.001$). The MAS assay detected 91 DRMs, while Sanger sequencing detected 86 DRMs, at the 20 DR loci. Thus, the two methods also showed excellent agreement in DRM detection at the individual-mutation level (kappa = 0.96; $P = 0.02$) (Table 1).

In this study, we demonstrated that the ASPE primers designed for subtype C MAS assay could be modified and optimized to detect DRMs in HIV-1 subtype B viruses, and the results were comparable to those of a validated Sanger sequencing assay (9). Low-abundance mutations in patient specimens were detected by the MAS assay but missed by Sanger sequencing. The major advantages of the MAS assay are straightforward result interpretation and high throughput (5). Unlike common sequencing methods, allele-specific assays directly detect each targeted mutation. Thus, the results are easy to interpret without the need for sophisticated analysis, for which the expertise is not always available in resource-limited settings.

Because of high levels of variation among HIV-1 sequences, even multiple degenerate bases are incorporated into ASPE primers to accommodate polymorphisms adjacent to the targeted alleles, but there are still low or no signals at a few DR loci in a small number of specimens because of mismatches between ASPE primers and viral sequences. Therefore, the MAS assay is not recommended for use alone for individual patient management. Nevertheless, the MAS assay was able to identify a great majority of the DRMs detected by Sanger sequencing and predicted the same number of DRMs at the patient level. This indicates that the accuracy of the MAS assay allows rapid estimation of DRM prevalence at the population level and represents a new tool for large-scale surveillance and routine monitoring of HIVDR.

ACKNOWLEDGMENTS

This project has been supported by the President's Emergency Plan for AIDS Relief (PEPFAR) through the U.S. Centers for Disease Control and Prevention and the appointment of Guoqing Zhang to the 2011-2012 International Emerging Infectious Diseases (EID) Fellowship Program sponsored by the Association of Public Health Laboratories (APHL) and the Centers for Disease Control and Prevention. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We sincerely thank all of the local staff in Honduras who participated in this study.

Chunfu Yang, Feng Gao, Guoqing Zhang, and Fangping Cai are the inventors in U.S. patent application US20140106977 A1. This does not alter the authors' adherence to all of this journal's policies on the sharing of data and materials.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Feng Gao under grant number RO1 GM065057.

REFERENCES

1. **Joint United Nations Programme on HIV/AIDS.** 14 July 2015. Fact sheet: 2014 global statistics. UNAIDS, Geneva, Switzerland. http://www.unaids.org/sites/default/files/media_asset/20150714_FS_MDG6_Report_en.pdf.
2. **Mann M, Lurie MN, Kimaiyo S, Kantor R.** 2013. Effects of political conflict-induced treatment interruptions on HIV drug resistance. AIDS Rev **15:**15–24.
3. **World Health Organization.** 2014. Surveillance of HIV drug resistance in adults receiving ART (acquired HIV drug resistance): concept note, July 2014. WHO, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/112801/1/9789241507073_eng.pdf.
4. **Dunbar SA.** 2006. Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta **363:**71–82. http://dx.doi.org/10.1016/j.cccn.2005.06.023.
5. **Zhang G, Cai F, Zhou Z, DeVos J, Wagar N, Diallo K, Zulu I, Wadonda-Kabondo N, Stringer JS, Weidle PJ, Ndongmo CB, Sikazwe I, Sarr A, Kagoli M, Nkengasong J, Gao F, Yang C.** 2013. Simultaneous detection of major drug resistance mutations in the protease and reverse transcriptase genes for HIV-1 subtype C by use of a multiplex allele-specific assay. J Clin Microbiol **51:**3666–3674. http://dx.doi.org/10.1128/JCM.01669-13.
6. **Song Y, Roumagnac P, Weill F-X, Wain J, Dolecek C, Mazzoni CJ, Holt KE, Achtman M.** 2010. A multiplex single nucleotide polymorphism typing assay for detecting mutations that result in decreased fluoroquinolone susceptibility in Salmonella enterica serovars Typhi and Paratyphi A. J Antimicrob Chemother **65:**1631–1641. http://dx.doi.org/10.1093/jac/dkq175.
7. **Junqueira DM, de Medeiros RM, Matte MC, Araujo LA, Chies JA, Ashton-Prolla P, Almeida SE.** 2011. Reviewing the history of HIV-1: spread of subtype B in the Americas. PLoS One **6:**e27489. http://dx.doi.org/10.1371/journal.pone.0027489.
8. **Diallo K, Lehotzky E, Zhang J, Zhou Z, de Rivera IL, Murillo WE, Nkengasong J, Sabatier J, Zhang G, Yang C.** 2014. Evaluation of a dried blood and plasma collection device, SampleTanker(®), for HIV type 1 drug resistance genotyping in patients receiving antiretroviral therapy. AIDS Res Hum Retroviruses **30:**67–73. http://dx.doi.org/10.1089/aid.2013.0127.
9. **Zhou Z, Wagar N, DeVos JR, Rottinghaus E, Diallo K, Nguyen DB, Bassey O, Ugbena R, Wadonda-Kabondo N, McConnell MS, Zulu I,**

Chilima B, Nkengasong J, Yang C. 2011. Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. PLoS One **6:**e28184. http://dx.doi.org/10.1371/journal.pone.0028184.

10. **Yang C, McNulty A, Diallo K, Zhang J, Titanji B, Kassim S, Wadonda-Kabondo N, Aberle-Grasse J, Kibuka T, Ndumbe PM, Vedapuri S, Zhou Z, Chilima B, Nkengasong JN.** 2010. Development and application of a broadly sensitive dried-blood-spot-based genotyping assay for global surveillance of HIV-1 drug resistance. J Clin Microbiol **48:**3158–3164. http://dx.doi.org/10.1128/JCM.00564-10.