ORIGINAL ARTICLE

Performance and Quality Assurance of Genotypic Drug-Resistance Testing for Human Immunodeficiency Virus Type 1 in Japan

Seiichiro Fujisaki, Saeko Fujisaki, Shiro Ibe, Tsukasa Asagi, Yoshihiro Itoh, Shigeru Yoshida, Takao Koike, Masayasu Oie, Makiko Kondo, Kenji Sadamasu, Mami Nagashima, Hiroyuki Gatanaga, Masakazu Matsuda, Mikio Ueda, Aki Masakane, Mami Hata, Yasushi Mizogami, Haruyo Mori, Rumi Minami, Kiyomi Okada, Kanako Watanabe, Takuma Shirasaka, Shinichi Oka, Wataru Sugiura, and Tsuguhiro Kaneda*

National Hospital Organization Nagoya Medical Center, Aichi 460-0001; Sendai Medical Center, Miyagi 983-8520; Department of Health Sciences, Hokkaido University School of Medicine, Hokkaido 060-0812; Department of Medicine II, Hokkaido University School of Medicine, Hokkaido 060-8638; Department of Virology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510; Division of Microbiology, Kanagawa Prefectural Institute of Public Health, Kanagawa 253-0087; Division of Virology, Department of Microbiology, Tokyo Metropolitan Institute of Public Health, Tokyo 169-0073; AIDS Clinical Center, International Medical Center of Japan, Tokyo 162-8655; AIDS Research Center, National Institute of Infectious Diseases, Tokyo 208-0011; Hematology Immunology, Ishikawa Prefectural Central Hospital; Ishikawa Prefectural Central Hospital (Japanese Foundation for AIDS Prevention), Ishikawa 920-8530; Department of Microbiology, Aichi Prefectural Institute of Public Health, Nagoya 462-8576; AIDS Medical Center, National Hospital Organization Osaka National Hospital, Osaka 540-0006; Division of Virology, Osaka Prefectural Institute of Public Health, Osaka 537-0025; Division of Immunology and Infectious Disease, Clinical Research Institute, National Hospital Organization Kyushu Medical Center; Fukuoka 810-8563; KITASATO-OTSUKA Biomedical Assay Laboratories Co., Ltd., Kanagawa 228-8555; and Section of Virus, Niigata Prefectural Institute of Public Health and Environmental Sciences, Niigata 950-2144, Japan

(Received November 27, 2006. Accepted January 23, 2007)

SUMMARY: Highly active antiretroviral therapy (HAART) can suppress human immunodeficiency virus type 1 (HIV-1) replication and plasma HIV-1 to below detectable levels. However, HAART becomes ineffective when drug-resistant viruses emerge during HAART. Monitoring drug-resistance mutations in viruses is necessary for selecting new drugs or therapies effective at inhibiting such HIV-1 variants. Most laboratories in Japan perform the tests using in-house protocols. However, the quality of these tests has never been assessed. Our study assessing the accuracy and reliability of HIV-1 genotypic drug-resistance testing in 15 laboratories in Japan revealed that the quality was very high (97.3% accurate). The errors, though rare, were caused by human errors, poor electropherograms, and the use of inadequate primers. Here, we propose troubleshooting procedures to improve testing accuracy and reliability in Japan.

INTRODUCTION

Genotypic drug-resistance testing for human immunodeficiency virus type 1 (HIV-1) is clinically useful for successful antiretroviral treatment (1-5). In Japan, the test, which could initially be performed at only a few virological laboratories in 1996, is now conducted at more than 15 laboratories. The cost of testing is almost entirely covered by government research funds. The laboratories use in-house protocols, the main advantage of which is their low cost relative to that of commercial kits. As the quality of these protocols had not previously been evaluated, we conducted an assessment of in-house protocol reliability by sending HIV-1 RNA samples to the relevant laboratories.

MATERIALS AND METHODS

Laboratories participating in the quality assurance assessment of genotypic drug-resistance testing protocols: Eight HIV/AIDS clinical centers, five local government institutes of public health, one commercial laboratory, and the National Institute of Infectious Diseases (NIID) were enrolled in this study on voluntary basis.

Construction of drug-resistant HIV-1 clones: We chose two plasma specimens, i.e., viruses well-characterized for harboring multidrug-resistance mutations. The HIV-1 of one specimen (case 1) had eight drug-resistance mutations: L63P, A71T, and V77I in the protease (PR), and A62V, V75I, F77L, F116Y, and Q151M in the reverse transcriptase (RT); and 10 mutations unrelated to drug-resistance: V3I, E35D, S37N, R41K, and K70R in the PR, and S68G, T69V, E122K, Q197E, and R211K in the RT. The HIV-1 of the other specimen (case 2) had nine drug-resistance mutations: L10I, L63P, V77I, and L90M in the PR, and M41L, T69S-SG insertion, G190A, L210W, and T215Y in the RT; and 15 mutations unrelated to drug-resistance: V3I, I15V, S37D, and I93L in the PR, and...

HIV-1 RNA was extracted from the plasma using the QIAamp Viral RNA kit (QIAGEN, Valencia, Calif., USA). DNA fragments sized 1.3 kb containing gag (codons #412-500), protease (codons #1-99), and reverse transcriptase (codons #1-260) were amplified by RT-PCR using SuperScript One-Step RT-PCR (Invitrogen, Carlsbad, Calif., USA) and the primer pair K1 (5'-AAG GGC TGT TGG AAA TGT GG-3') and U13 (5'-CCC ACT CAG GAA TCC AGG T-3'), followed by a second-round PCR using LA Taq (Takara, Shiga, Japan) with the primer pair INF-Apal (5'-TGC TGG GCC CCT AGG AAA AAG G-3') and INF-NheI (5'-TCT GGC TAG CCC AAT TCA ATT TTC CCA C-3'). The amplified fragments were sequenced and the presence of the target mutations was confirmed. The amplicon was then digested with NheI (New England Biolabs, Ipswich, Mass., USA), and the Inf-NheI fragment was inserted into the corresponding site of a pSUM9 HXB2 expression vector (kindly provided by Dr. H. Mitsuya, National Cancer Institute, Bethesda, Md., USA) (6). Each clone was transfected with RPMI 1640 containing 10% FBS under a 5% CO2 until use. The culture supernatants were harvested and stored at –80°C until use.

Preparation and shipping of HIV-1 RNA samples: HIV-1 virions were precipitated by ultracentrifugation at 23,000 x g for 1 h at 4°C and washed twice with PBS(-). The pellets were suspended in 100 μl of PBS(-) and incubated with 3 U of deoxyribonuclease (RT Grade) (Nippon Gene, Tokyo, Japan) at 37°C for 15 min. Virions were precipitated again by ultracentrifugation at 23,000 x g for 1 h at 4°C and washed twice with PBS(-) to remove the deoxyribonuclease. RNA was extracted using the QIAamp Viral RNA kit. The number of HIV copies was determined by real-time PCR according to the method reported by Nagai et al. (7), and aliquots containing 2 x 10^6 copies of HIV-1 RNA in 2 ml of PBS(-) were stored at –80°C. The samples were packed on dry ice and shipped to the participating laboratories.

Data collection and evaluation: Each laboratory was requested to provide a detailed protocol for genotypic testing, including the primer sequences and the enzymes used for reverse transcription and PCR conditions. Laboratories were asked to submit electropherograms and a list of drug-resistance mutations determined according to International AIDS Society-USA panel criteria, version March/April 2005 (8).

The rate of successful detection of mutations was expressed as follows: %DR (or NDR) = detected number of drug-resistance mutations (non-drug-resistance mutations)/total number of drug-resistance mutations (non-drug-resistance mutations). Reported mutations absent in the test samples (ghost mutations) were noted as “E1” errors, erroneous categorization as an “E2” error, and errors in preparing the reports as “E3” errors.

RESULTS

Variations in protocols for genotypic drug-resistance testing in 15 laboratories: As shown in Figure 1, the enrolled laboratories used different protocols with respect to primers, sizes of amplified fragments, and the number of amplified fragments. Eight laboratories (A, B, D, E, F, I, J, and K) used the latest NIH protocol with minor modifications, and three laboratories (G, H, and N) used the 1996 version of the NIH protocol with or without modifications. Four laboratories (C, L, M, and O) used their own protocols.

Regarding the reverse transcription and amplification procedures, 12 laboratories used one-step RT-PCR, and the other three laboratories used two-step RT-PCR. In the reverse-transcription process, AMV RT was used in two laboratories, and M-MLV RT was used in one laboratory. As regards the DNA polymerase used for the first and second PCR, most laboratories used Taq polymerase but other few laboratories used KOD polymerase.

For sequencing, Big Dye Terminator V1.1 (Applied Biosystems, Foster City, Calif., USA), Big Dye Terminator V3.1 (Applied Biosystems), CEQ Dye Terminator Cycle Sequencing with a Quick Start kit (Beckman Coulter, Fullerton, Calif., USA), and Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, Ohio, USA) with IRDye™ 800 v2 Terminator Mixes (LI-COR, Lincoln, Nebr., USA) were used in eight, five, one, and one of the laboratories, respectively. For the purification of labeled products, CENTRI SEP Spin Columns (Applied Biosystems), Sephadex G-50 (GE Healthcare Bio-Sciences, Piscataway, N.J., USA), and DyeEx 2.0 Spin kit (QIAGEN) were used in five, three, and one of the laboratories, respectively. The other six laboratories used the ethanol precipitation method.

For electrophoresis, 14 laboratories used a capillary-type auto-sequencer and the remaining laboratory used a plate-type auto-sequencer. Ten laboratories used the ABI PRISM 310 (Applied Biosystems) auto-sequencer and five laboratories used other sequencers, i.e., the CEQ 8000 (Beckman Coulter), LI-COR 4200 IR2 System (LI-COR), ABI PRISM 3100 (Applied Biosystems), ABI PRISM 3100 Avant (Applied Biosystems), and ABI PRISM 3730S (Applied Biosystems) auto-sequencers.

Results of case 1 testing: As shown in Table 1, in the PR mutation analyses, all laboratories except G detected 100% of the drug-resistance mutations. On the other hand, when detecting the non-drug-resistance mutations, the amino acid mutations reported by laboratories G and H were totally different from those reported by the other laboratories. Laboratory G missed one drug-resistance mutation, A71T, and two non-drug-resistance mutations, E35D and R41K. Laboratory H reported all of the three drug-resistance mutations correctly, but not all of the five non-drug-resistance mutations. Laboratories G and H appear to have amplified the wrong samples, suggesting that contamination may have occurred. Checking their protocols, we noticed that laboratories G and H used DRPRO3 primer in their second round of PCR (Figure 1). As the case 1 sample had an insertion mutation in the DRPRO3 annealing region, a mismatch with this primer in the region caused these errors.

Laboratories E and F reported ghost mutations D29N and C95W, respectively. In the case of laboratory E, the error appeared to be due to the high background noise in the electropherogram. In the case of laboratory F, the data were analyzed only by automatic base sequence analysis, and no manual editing was performed to eliminate the error.

Two laboratories (A and B) made errors in their final reports. We confirmed the raw data from these two laboratories, and found that their electropherograms, nucleotide
sequences, and amino acid sequences were correct, but that they misrecorded the amino acid mutations during the preparation of their final reports.

As regards the RT results, 14 laboratories correctly reported all of the drug-resistance and non-drug-resistance mutations. Laboratory C did not report three non-drug-resistance mutations (S68G, Q197E, and R211K), although these were correctly displayed in the nucleotide and amino acid sequence files, thus indicating an error in the preparation of the final report.

**Results of case 2 testing:** In the PR mutation analysis, 12 laboratories correctly reported all of the drug-resistance and non-drug-resistance mutations. Laboratory D failed to report the L90M drug-resistance mutation owing to a translation error, although “ATG” was clearly seen in the electropherogram. Laboratory E reported ghost mutation E34K due to the high background noise in the electropherogram. Peak distortion on the electropherograms caused Laboratory F to report ghost mutations R41K and T96S, and an extra large peak on the electropherogram caused the misdetection of L10I/S instead of L10I. This extra large peak may be due to the insufficient removal of residual free dideoxynucleotides after the sequence reaction.

As regards the RT region, 14 laboratories, but not laboratory K, correctly reported all drug-resistance mutations. Laboratory K detected the T69S-SG insertion, but misidentified it as a non-drug-resistance mutation. As for the non-drug-resistance mutations, four laboratories (G, H, L, and N) prepared incorrect reports. Laboratory G did not report V35T and T39A because they were unable to analyze the region between codon #1 and #39. Laboratory H did not report E122K and I135T, because they did not amplify the RT region from codon #122 to #135. These errors reported from laboratories G and H were excluded in the calculation of the rate of detection of non-drug-resistance mutations (%NDR). Laboratory L failed to report K238K and I31T, although it was detected in the amino acid sequence file. Laboratory N reported ghost mutation I31T.

**DISCUSSION**

To assess the quality of in-house genotypic drug-resistance testing, we used two HIV-1 RNA specimens to conduct profi-
ciency tests for 15 voluntarily participating laboratories. Since there were 42 amino acid mutations within the sequences of the two test samples, 13 of the laboratories should have reported 546 amino acid mutations and the remaining two (G and H) should have reported 80. Accordingly, the total number of amino acid mutations reported from 15 laboratories should have been 626. There were a total of 17 errors in their reports. The calculated overall detection percentage was 97.3% (= 100\[\frac{529}{626}\]), and the error rate was 2.7%.

The average correction rate of eight laboratories (A, B, D, E, F, I, J, and K) adopting the NIID protocol was 97.0% and 97.6%, respectively. Thus, there was no obvious benefit of in-house protocols in terms of accuracy. However, the in-house protocols of L, M, and O possessed one superior point where the single DNA fragment including PR and RT genes were amplified. Based on these results, we can propose that the NIID protocol be considered as the standard protocol. As the primers developed by laboratories L, M, and O functioned well, we can recommend the usage of these primers in the standard protocol.

The errors observed in this study were categorized as either technical or human errors, and the frequency of these errors was 1.4 and 1.3%, respectively. As regards the technical errors, we found two problems that need to be addressed. First, the use of mismatched primers led to errors in the detection of case 1 HIV-1 PR mutations. The case 1 sample had an insertion at the annealing site of the DRPRO3 primer, and the laboratories using that primer (G and H) failed to detect the mutations. A new primer, DRPRO1M, replaced DRPRO3 when the insertion mutation at #122 to #135, respectively.

Second, the quality of the primers appeared to be important. Using impure primers may result in high background noise on the electropherogram, which may lead to the misinterpretation of results. Therefore, using highly purified primers is recommended for better sequence reliability. Needless to say, the buffers, matrix, and capillaries of the sequencers should always be in good condition.

Several types of human error were observed, including errors in writing or copying the final reports (1%), errors in categorizing mutations as resistance or non-resistance mutations (0.2%), and misinterpretation of codons (0.2%) (Table 2). The ideal way to prevent such human errors would be the development of a computation program that would automatically analyze electropherograms, identify drug-resistance mutations, and create the final report. Until such resources are available, several manual validation steps will be necessary to check the data and the content of the reports.

As regards the detection of different HIV-1 subtypes, both the latest NIID protocol and the in-house protocol of laboratory O have successfully amplified the PR and RT genes of HIV-1 subtypes A, B, C, D, AE, and F.

In conclusion, we are pleased to report that the reliability of genotypic drug-resistance testing in Japan is excellent (97.3% correct results). However, as noted above, there are a few points related to implementation which still need to be improved upon in order to achieve even better results. We have sent out the annotated results of our evaluation to each laboratory, and hope that this report will help to improve the
quality of testing. Furthermore, as the test samples used here were extracted viral RNAs and not the virus itself, the technical error expected with the RNA extraction procedure was not considered in the present assessment. Thus, the error rate of 2.7% may be an underestimation; to clarify the exact rate, we are currently planning a second round of assessment using patient plasma samples.

ACKNOWLEDGMENTS

We thank Dr Hiroshi Yoshikura for his critical reading of our manuscript. We also thank Ms Clair Brown for her help in preparing the manuscript. This study was supported by a Research Grant for Research on HIV/AIDS from the Ministry of Health, Labour, and Welfare of Japan (No. H-16-AIDS-002).

Seiichiro Fujisaki and Saeko Fujisaki are research residents of the Japanese Foundation for AIDS Prevention.

REFERENCES