



Performance of the TRUGENE HIV-1 Genotyping Kit and the Applied Biosystems' HIV-1 Genotyping System in Sequence-Based Analysis of non-B HIV-1 Subtypes.

L. Jagodzinski*, J. Cooley¹, S. Kelly¹, N. Michael².

¹Henry M. Jackson Foundation, and ²WRAIR, U.S. Military HIV Research Program, Rockville, MD.



Correspondence to: Linda Jagodzinski, 1600 E. Gude Drive, Rockville, MD 20850, Ph: 301-251-5042, Fax: 301-762-7460, e-mail: ljagodzinski@hivresearch.org

ABSTRACT

Background: Sequence-based genotypic assays that determine drug resistance mutations in the HIV genome are increasingly being used to make changes in patients' drug regimens. The Visible Genetics' TRUGENE HIV-1 Genotyping Kit is the first sequence-based assay to receive FDA approval for use in patient management, making HIV-1 genotyping analysis a standard-of-care test in HIV-1 treatment decisions. Given the diversity of HIV-1 subtypes, the emergence of recombinant HIV-1 subtypes and the emergence of non-B subtypes in the United States, genotypic assays must be able to deliver sequence data on diverse HIV-1 subtypes. A panel of thirty-four HIV-1 viral isolates representing HIV-1 subtypes A through H was used to assess the performance of two commercially manufactured assays: Visible Genetics' TRUGENE HIV-1 Genotyping kit and Applied Biosystems' HIV Genotyping System (ViroSeq). Result: Visible Genetics' TRUGENE assay successfully genotyped 94.1 % (32/34) of the HIV-1 subtype panel. Sequence data was not obtained for one C and one G subtype isolate in the TRUGENE assay. Applied Biosystems' ViroSeq assay successfully genotyped 100% of the HIV-1 subtype panel. Comparison of nucleotide sequences generated by the two assays showed only minor differences that were related to single defined base versus mixed base calls. The inability of the TRUGENE assay to sequence two members of the HIV-1 subtype panel may indicate some problems in the amplification of the HIV-1 protease and reverse transcriptase gene regions. Clinical specimens when tested in both assays have failed in one or the other assay with a success rate of ~90% observed for both assays. Conclusion: The Visible Genetics and Applied Biosystems' HIV-1 Genotyping tests generated equivalent sequence data for all sequenced members of the HIV-1 subtype panel. Both tests are capable of delivering sequence data on diverse HIV-1 subtypes and detecting defined drug resistance mutations.

the U.S. deploys military troops, the ability of the HIV-1 genotypic assays to detect drug resistant mutation sites in non-B subtypes is essential in the treatment of military personnel infected with non-B subtype strains.

This study assesses the performance of the TRUGENE HIV-1 Genotyping Kit and the ViroSeq Genotyping System using a panel consisting 34 members encompassing HIV-1 subtypes A through H.

METHODS

Samples.

A panel of 34 HIV-1 isolates of Group M subtypes A through H was used to assess the performance of the TRUGENE and ViroSeq HIV-1 Genotyping tests. The HIV subtype was determined for each panel member in previous studies using in-house sequencing procedures. Viral stocks were previously standardized by particle count^{1,2}. Each viral stock was diluted into normal human plasma to generate viral dilutions targeted at 10,000-30,000 and 100,000-300,000 RNA copies/ml. HIV viral RNA concentrations were determined using the Roche Amplicor HIV-1 MonitorTM Test, Version 1.5. Viral stocks at concentrations less than 10,000 RNA copies/ml were not tested since the goal of this study was to determine the ability of each assay to generate amplicon and sequence data for the protease-reverse transcriptase gene regions. Lower limit sensitivity was not an issue for this study. Twenty-nine of the viral stocks were from the Walter Reed Army Institute of Research HIV subtype panel. Five of these viral stocks were obtained from BBI-Biotech and are members of the NIAID HIV subtype panel.

Genotypic Resistance Testing.

Scientists trained and well versed in the execution of the Visible Genetics' and Applied Biosystems' tests performed both assays. RNA was extracted from each member of the panel following extraction procedures recommended by the manufacturer of each assay. Panel members that failed to generate sequence data by either method were repeated in the entirety by another certified technologist.

1. TRUGENE HIV-1 Genotyping Kit - RNA was extracted from 0.14 ml of each panel member using the QIAampTM Viral RNA Extraction Kit following the product insert. Purified RNAs were tested using the TRUGENE HIV-1 Genotyping Kit and OpenGeneTM DNA Sequencing System according to the Manufacturer's instructions.

2. ViroSeq HIV-1 Genotyping Kit - RNA was extracted from 0.5 ml of each panel member and tested using the ViroSeq HIV-1 Genotyping System, Version 2.0. Sequencing reaction products were fractionated using Applied Biosystems' 377 XL Genetic Analyzer. Sequencing results were analyzed using the ViroSeq HIV Genotyping System Software (Version 2.2: Applied Biosystems, Inc.), and SequencerTM Version 3.1 (Gene Codes Corporation, Ann Arbor, Mich.). Two procedural differences are noted for the ViroSeq assay.

i) The concentration of the purified PCR product was determined by extrapolation of the value from a standard curve generated by densitometry for a DNA mass ladder. If less than 15 nanograms/5ul of amplicon was obtained, insufficient PCR product was available for sequencing in the ViroSeq assay.

ii) The ViroSeq assay requires purification of the sequencing reactions followed by vacuum drying of the reactions prior to

fractionation by polyacrylamide or capillary electrophoresis. Edge Biosystems' 96-well separation plates were used to purify DNA sequencing reactions.

Analysis.

Sequence contigs were generated for each panel member using software provided by the manufacturer of each test. All highlighted discrepancies were reviewed and the sequences edited. The nucleotide sequences generated for each member of the panel were aligned using the MegAlign program of Lasergene Navigator, Version 1.6. (DNASTAR Inc., Madison, Wis.). Individual base differences were noted and reconfirmed by looking at the sequence data generated by both assays. Corrections were made if an error was found in a base call. All sequences were checked for uniqueness and subtype grouping by Phylogenetic Tree analysis using neighbor joining, maximum likelihood, and distance analyses (Wisconsin PackageTM, Version 10: Genetics Computer Group).

RESULTS

Performance of the ViroSeq Test.

All 34 viral isolates (2 subtype A, 2 subtype A/G, 7 subtype B, 6 subtype C, 2 subtype D, 8 subtype E, 4 subtype F, 2 subtype G, and 1 subtype H) were amplified and sequenced using the Applied Biosystems' ViroSeq Genotyping System, Version 2.0. At least 18 ng of amplicon was measured in five microliters of purified PCR product for each of the panel members. Failed sequencing primers are shown in Table 1. Primer D failed to generate sequence data for 22 of the panel members. This number represents a 65% failure rate. Primers A, F, and G failed on 3 specimens each for a failure rate of 9% each. Poor sequencing results were observed for primers A, B, D, G, and H for 3, 5, 2, 7, and 6 panel members, respectively. Since primer D is a backup for primer A, two of the panel members had single-strand sequences in the protease gene. The sequence for panel member US3 was poor due to either primer performance or to fractionation through the polyacrylamide gel. This panel member is being repeated with fractionation on the 3100CE.

Performance of the TRUGENE Test.

The Visible Genetics' TRUGENE HIV-1 Genotyping test successfully sequenced 31 out of 34 panel members. One C and one G subtype failed multiple times in the assay using the lower 10,000-30,000 RNA copy/ml dilution and the dilution targeted at greater than 100,000 RNA copies/ml. Sequence data generated for the other G subtype (BCF-DIOUM) was of poor quality and repeat testing is in progress. The PR primers failed in 23 of the specimens having a failure rate of 75%. In general, the PR primers worked well on subtype B isolates, while the P2 primers performed better on all of the other subtypes. Single strand sequence data was generated for a short region at the beginning of the protease gene when sequence data was obtained using only the P2 primers. Single-strand sequence regions in the reverse transcriptase gene are noted for four of the E subtypes (POC30506, ID12, ID17, and NP1465), three of the F subtypes and the single H subtype.

Comparison of Sequence Data.

The sequences generated for each panel member by both HIV-1 Genotyping assays were aligned using MegAlign program. Base calls which differed were rechecked for accuracy and corrected if found to be incorrect. Ninety-seven percent of the differences noted were due

to differences in ambiguity calls where sites are called as a mixture of two or more bases versus single base calls. Of 58 site differences, 36 of the differences were due to ambiguities in the TRUGENE reading, where one of the calls in the mixed base call was equivalent to the single base call given in the ViroSeq assay. The ViroSeq test accounted for 20 ambiguities that were in agreement with the defined base call given by the TRUGENE test. Two of the scored differences were due to different base calls between the two assays. These calls were not located at drug resistance sites and did not alter the interpretation of the resistance read. Fourteen (45%) of the sequences generated by both assays were identical.

Table 1: Summary of Sequencing Results for 34 Member Subtype Panel.

Table with 10 columns: HIV-1 Subtype, Isolate ID, Country, TRUGENE (Sequence, Failure), Comments, ViroSeq (Sequence, Failure), Comments. Rows include subtypes A, B, C, D, E, F, G, and H with various isolate IDs and countries.

* Sequence obtained was of poor quality but readable. Needs to be repeated. ** Sequence obtained did not meet quality control measures. Needs to be repeated.

Observations.

The TRUGENE test generates a research report that lists all of the drug resistance mutation sites, silent mutations, polymorphisms, and unexpected mutations. This report could be used to identify non-B subtypes. Table 2 shows the average number of silent mutations observed in seven of the subtypes for the protease and reverse transcriptase gene regions. All of the non-B subtypes have significantly larger numbers of silent mutations. D subtype has the lowest number of silent mutations next to the B subtype. A long list of silent mutations may suggest that the sequence obtained for a clinical specimen is a non-B.

Table 2: Number of Silent Mutations Observed for Subtype Panel.

Table with 3 columns: Subtype, Average Number of Silent Mutations Protease / RT, Average Differences as Compared to B-Subtype Strains. Rows include subtypes A, B, C, D, E, F, H*.

* Only one specimen included for this subtype. Subtype D has the least amount of silent mutations next to subtype B.

Resistance Sites Detected.

The HIV-1 subtype panel used in this study consisted of 34 HIV isolates that have been cultured and characterized by p24 antigen testing, particle counting by electron microscopy and viral RNA testing^{1,2}. These viral isolates were cultured from clinical specimens obtained from drug naive individuals. As such these isolates would have few drug associated resistance sites. Table 3 shows the sites detected by both of the HIV-1 resistance genotyping tests. The majority of the resistance sites were located in the protease gene. Most of these sites are designated as secondary resistance sites and do not confer resistance without the presence of a primary site. M46L is both a primary and secondary resistance site and was found in 2 of the E subtype isolates.

Table 3: Drug Associated Mutations Observed in HIV Subtype Panel.

Table with 2 columns: Mutations In Protease, Subtype (s). Rows include L101V, K20R, M36I, M46/M or M/L, L63P, V77I.

Two resistance mutations were observed in the reverse transcriptase gene: A98S in two C subtype panel members and I50I/V in one C subtype strain. The I50I/V mutation is noted as being found in virus obtained from culture.

CONCLUSIONS

- 1. The Applied Biosystems' ViroSeq Genotyping System and Visible Genetics' TRUGENE HIV Genotyping Kit are both capable of delivering HIV-1 resistance data for clinical specimens infected with non-B subtype virus. 2. The ViroSeq assay generated sequence data for all 34 members of the panel. Amplification was excellent for this assay based upon the amount of RT-PCR product generated for each viral isolate. Performance issues exist for the sequencing primers where regions of single-strand sequences are generated due to poor primer performance or in regions where sequence data had high backgrounds making it difficult to read. Problems encountered in polyacrylamide gel electrophoresis may be eliminated through the use of 3100 capillary electrophoresis instrumentation (3100CE). 3. The TRUGENE assay generated sequence data for 31 of the subtype panel members. Performance issues exist for the primers pairs used in sequencing in the reverse transcriptase region for E, F, and H subtypes since regions of single strand sequence data were obtained for these isolates. Of concern is whether the G subtype can be sequenced by the TRUGENE assay. 4. Equivalent sequence data is generated by the ViroSeq and TRUGENE assays. Differences observed are due to ambiguities resulting in mixed base sequence calls in one assay versus defined single base calls in the other assay system. All of these mixed base calls are in agreement with the single base calls given in the other assay system. 5. Improvements in both assays are required in order to ensure that double strand sequence data is generated for all of the HIV-1 subtypes. Primer design is critical in amplification and sequencing of diverse subtype sequences.

References

- 1. Michael, N.L., Herman, S.A., Kwok, S., Dreyer, K., Wang, J., Christopherson, C., Spadaro, J.P., Young, K.K.Y., Polonis, V., McCutchan, E.E., Carr, J., Mascola, J.R., Jagodzinski, L.L., Robb, M.L.: Development of calibrated viral load standards for group M subtypes of human immunodeficiency virus type 1, and the performance of an improved Amplicor HIV-1 Monitor Test with isolates of diverse subtypes. J. Clinical Micro. 37: 2857-2863, 1999. 2. Jagodzinski, L.L., Wiggins, D.L., McManis, J.L., Emery, S., Overbaugh, J., Robb, M., Bodrug, S., Michael, N.L.: Use of calibrated viral load standards for group M subtypes of human immunodeficiency virus type 1 to assess the performance of viral RNA quantitation tests. J. Clinical Micro. 38: 1247-1249, 2000.

INTRODUCTION

The human immunodeficiency virus (HIV-1) population is continuously evolving, generating genetically distinct viral variants. The propagation of novel mutants is a function of environmental selective pressures created by immunological responses of the host and drug therapy. Thus, mutants that confer a survival advantage will propagate and emerge as a major quasispecies. The incomplete inhibition of HIV replication can lead to the emergence of virus that has reduced susceptibility to antiretroviral drugs, limiting the efficacy of antiretroviral treatment regimens. Drug resistant HIV-1 can also be transmitted from one individual to another. Mutation sites conferring resistance have been identified for most of the antiretroviral drugs and can be determined through genotypic analysis of HIV-1 viral RNA.

The determination of drug associated mutations is accomplished using automated DNA sequencing methodologies. Two sequence-based assays are available commercially for use in the detection and identification of HIV-1 drug resistance sites in clinical specimens. The Visible Genetics' TRUGENETM HIV-1 Genotyping Kit has FDA approval for use in detecting HIV genomic mutations that confer resistance to antiretroviral drugs and as an aid to physicians in monitoring and treating HIV infection. The Applied Biosystems' ViroSeqTM Genotyping System is presently in review by the FDA. Both assays were originally designed and optimized for subtype B strains. Since non-B subtypes predominate in other regions of the world where