CELERA RUO INTEGRASE RESISTANCE ASSAY PERFORMS WELL ACROSS SEVERAL SUBTYPES

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BACKGROUND

Global access to antiretroviral therapy (ART) has increased rapidly over the last ten years in developing countries, where over 10 million infected individuals are now receiving ART. ART can fail as a result of toxicity, transmitted drug resistance, inadequate medication adherence or incomplete suppression of viral replication, resulting in the emergence of drug-resistant HIV-1. When HIV drug resistance develops, ideally a new regimen containing at least three active drugs should be administered. Optimal third-line options are still being investigated in studies such as the "Management Using the Latest Technologies in Resource-limited Settings to Optimize Combination Therapy After Viral Failure (MULTI-OCTAVE)" a multicenter international trial of the AIDS Clinical Trials Group (ACTG). This study includes the drug raltegravir from the integrase class of inhibitors.

Raltegravir inhibits the integrase enzyme, thus preventing the integration of HIV cDNA into the host genome. Decreased susceptibility to raltegravir develops as a result of several different mutations in the integrase region. To date, the majority of work in detecting integrase resistance has centred on HIV subtype B. However, the subtypes circulating in developing countries, where the need for treatment is greatest, are generally non subtype B. The subtypes which predominate in these developing countries are subtype A, C, D and several recombinant forms. Sequence variations in the binding sites for PCR amplification primers and sequencing primers are a major obstacle to developing molecular based assays for this range of subtypes. One commercially available test for integrase resistance is available (ViroSeq™ HIV-1 Integrase RUO Genotyping Kit [Celera, US]).

In the current study we sought to independently assess the performance characteristics of ViroSeq™ HIV-1 Integrase RUO Genotyping Kit for patient samples with a broad range of subtypes.

METHODS

Patient Samples

One hundred and sixty four samples were used in the validation.

One hundred and six samples from integrase inhibitor naïve patients' plasma samples sent for routine HIV-1 drug resistance testing after failing a 1st- or 2nd-line regimen;

Fifty eight from an external virology quality assurance program (VQA).

Of the 58, 15 were identical VQA samples, which were tested in two different laboratories to assess assay reproducibility.

Population Genotype Analysis:

Population based genotyping was performed using the ViroSeq™ HIV-1 Integrase RUO Genotyping Kit (Celera, US). Viral RNA was extracted from plasma using the ViroSeq extraction module and a 1.1 kilobase amplicon was generated from a one-step RT-initiated polymerase chain reaction encompassing the entire integrase region. This amplicon was used as a template to sequence 0.9kb of the integrase region using four sequencing primers. Sequencing was performed with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, USA). If sequencing primers failed they were repeated. Sequences were analysed using the ViroSeq™ Integrase Software V1.0.0 or Sequencher v5.0 to give the drug resistance profile across codons 1-288 of integrase (Figure 1).

Data Analysis:

The Rega HIV-1 subtyping tool was used to designate the HIV-1 subtype of each sample. Integrase polymorphisms and mutations were determined using both the ViroSeq™ Integrase V1.0.0 algorithm and the Stanford database (HIValg program). Both algorithms gave drug penalty scores for raltegravir, elvitegravir and dolutegravir. A phylogenetic tree analysis was performed on the sequences generated from the two different testing laboratories to ensure all duplicate samples clustered together. To access reproducibility the paired samples from the two different laboratories had the nucleotide sequences aligned and the level of disagreements compared.

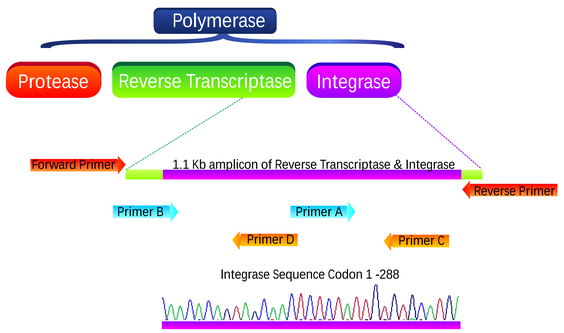


Figure 1: Diagrammatic representation of *pol gene* of HIV with the position of the amplicon and sequencing primers of the ViroSeq™ HIV-1 Integrase RUO Genotyping Kit

The level of disagreements was compared as follows a) number of sequencing differences (complete difference of nucleotide at the same point); b) the number of partial-mismatches (for example R vs. G). Hamming distance was calculated for each of the parameters evaluated and a distance of greater that 98% for complete mismatches was considered acceptable. Lastly, the mutation lists obtained through the Stanford HIValg Program were compared for sequences derived from both laboratories.

RESULTS

Amplification:

The ViroSeq™ HIV-1 Integrase RUO Genotyping Kit indicates that 1000 RNA copies/ml is the lower limit of detection. No samples with viral loads less than the reported limit of detection were tested. Of the 164 samples tested the HIV-1 RNA ranged from 3.15 to 6.74$_{log}$copies/ml with a mean viral load of 4.53$_{log}$copies/ml, successful amplification was obtained for 95% of samples (n=156).

Table 1: Overall performance of the ViroSeq™ HIV-1 Integrase RUO Genotyping Assay, by subtype

Subtype	No Amplification*	No Sequencing*	No Bi-directional Sequence	Full Sequence	Total
A1	2	4	1	17	24
D	3	1		12	16
Recombinant	2	1	1	24	28
C	1	7	12	41	61
B			3	27	30
F1			1	2	3
G				2	2
Total	8	13	18	125	164

*Subtype determined by sequencing of protease and reverse transcriptase.

The HIV-1 RNA of the samples that failed to amplify ranged across viral loads tested, indicating that viral load was not the main determinant of amplification success. Of the eight samples that failed to amplify, the subtypes (determined by sequencing of the reverse transcriptase and protease region) were subtype D (n=3), subtype C (n=1), CRF02_AE (n=1), subtype A1 (n=2), and unassigned subtype (n=1) (Table 1).

Sequencing:

Of the 156 samples that successfully amplified, no sequence could be generated for 13 (8%) of the samples, as all four sequencing primers failing to work. Of the 13 samples that failed to sequence, the subtypes were subtype D (n=1), subtype A1 (n=4), CRF01_AE (n=1) and subtype C (n=7; Table 2).

Table 2: Sequencing primers which failed, by subtype

Subtype (n=18)	Primer A	Primer B	Primer C	Primer D
A1 (n=1)				1
B (n=3)	1	1	1	
C (n=12)	1	5	2	5
CRF01_AE (n=1)			1	
F1 (n=1)	1			
Total	3	6	4	6

Sequencing was therefore successful in 143 samples, however, complete bidirectional sequencing was only obtained in 125 (78%) samples. Of the 18 samples (13%) that did not have full bidirectional sequence, Table 2 showed the sequencing primers that failed and integrase subtypes of these samples. Subtype C samples had the highest number of primer failures, resulting in incomplete bidirectional sequencing of the integrase region in 12 of 61 (20%). Failure of sequencing primer D resulted in no sequence for the first 40 codons of integrase.

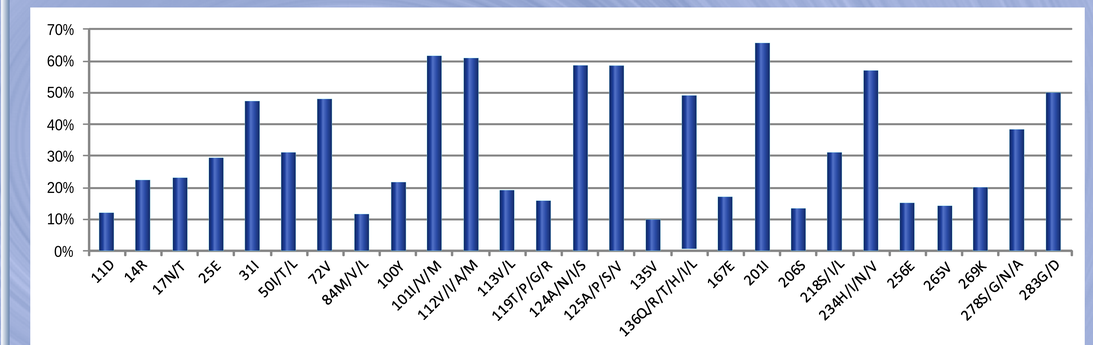


Figure 2: Integrase Polymorphisms in Integrase Naïve subjects

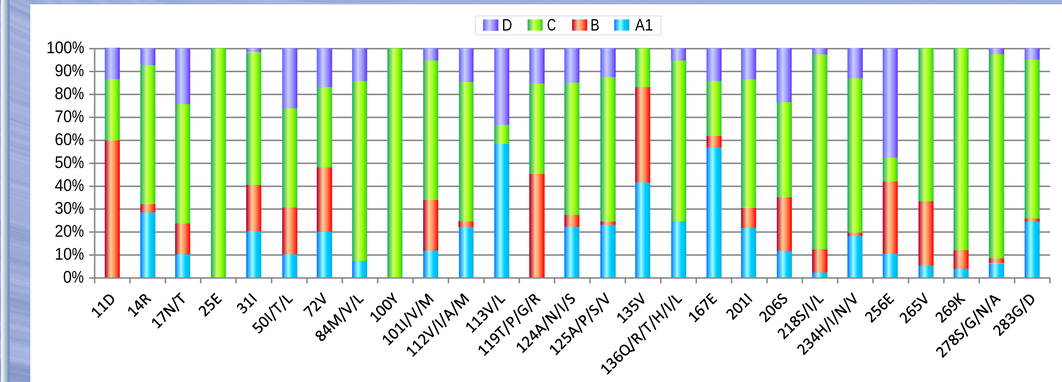


Figure 3: Integrase Polymorphisms in Integrase Naïve subjects, by subtype

Reproductibility:

Reproducibility of the ViroSeq™ HIV-1 Integrase RUO Genotyping assay was determined by processing 15 duplicate samples, supplied by the VQA, across two different laboratories. Laboratory 1 was able to successfully amplify all 15 samples whereas laboratory 2 was not able to amplify one of the 15 samples. Of the sequences obtained in both laboratories, sequence homology ranged from 99.01% to 100%. However, Laboratory 2 detected 23 mixtures; whereas Laboratory 1 only detected 9 mixtures in the 11219 nucleotides analysed, possibly as a result of differing methods of sequence analysis.

Integrase Mutations & Polymorphisms:

All the patient samples tested were raltegravir naïve; however, seven (5%) had mutations linked to reduced susceptibility to an integrase inhibitor as determined by the Stanford HIValg program. The following mutations were observed [Q148K; E138K; G140A (n=1)], T97A (n=2), L74I (n=4). Of the four samples with L74I, three were subtype G. Polymorphisms not linked to integrase resistance that occurred at greater than 10% overall are shown in Figure 2 and by subtype A, B, C and D in Figure 3. The majority of the polymorphisms were observed in HIV-1 subtype C.

CONCLUSION

Of the 164 samples tested, 76% (n=125) were successfully amplified and a complete bi-directional sequence obtained. Sequencing results were similar between two testing laboratories with the exception of mixture detection (more frequent in Laboratory 2 than 1). Mutations associated with integrase inhibitor resistance as determined by the Stanford HIValg program were observed in only 5% of integrase inhibitor naive samples, and most of these mutations are likely to be due to subtype-specific polymorphisms rather than selection by an integrase inhibitor, although further analysis of samples from before and after raltegravir therapy is needed.

Acknowledgements

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