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Single Base Primer Extension Assay (*SNaPshot*) for Rapid Detection of Human Immunodeficiency Virus - 1 Drug Resistance Mutations

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Abstract

The 3' primer extension assay can be employed to interrogate multiple known single nucleotide polymorphisms (SNPs) simultaneously using a multiplex approach. Over 200 Human Immunodeficiency Virus-1 (HIV-1) mutations are currently associated with drug resistance. Identifying these mutations assist in taking appropriate therapeutic decisions such that superior drug regimens can be designed for keeping the viral load below pathogenic level. Presently, reverse transcription of HIV-1 RNA followed by amplification of the *pol* gene and subsequent nucleotide sequencing is a popular method of HIV-1 drug resistance genotyping. However, it is still a time consuming and laborious protocol and require significant bioinformatics analysis in order to decipher the significance of mutations encountered during analysis.

In this study we analyzed 75 HIV-1 positive clinical samples for 2 representative mutations, *viz*., M41L and M184V using a 3' primer extension protocol popularly known as *SNaPshot* assay. Twenty four and 36% of the patients were found to harbor the critical M41L and M184V mutations respectively while 20% were found to contain both the mutations together. The results were 100% concordant with DNA sequencing assay which was used as a gold standard. The results substantiate the cost, speed and economy-related advantages of this technology platform for interrogating known mutations of significance within the HIV-1 genome.

Keywords: HIV-1; Drug resistance; *SNaPshot;* HAART; 3TC; ddI

Introduction

Oligonucleotide primers extend at their 3' end when annealed to a template in presence of functional DNA polymerase, dNTPs and buffer solution that impart a suitable environment for DNA replication [1]. This basic property has laid the foundation for a host of advanced DNA/RNA analysis techniques. The extension of primer involve hybridization of a probe to the template in a way that its 3' end is positioned one base before the target single base mutation that is to be interrogated. This is followed by an enzymatic reaction similar to fluorescent DNA sequencing where all the incorporating bases are dideoxynucleotides or ddNTPs and labeled with different dyes. The one that incorporates against the hot spot base is then identified by its color and the nature of mutation deciphered by logical conclusion [2].

This technology is versatile and can identify most of the SNPs under a multiplexed condition making the process highly flexible and adaptable. The process can be incorporated with multiple applications including MALDI-TOF Mass spectrometry and ELISA-like methods [3]. Therefore, the primer extension protocol is capable of genotyping most SNPs under very similar reaction conditions making it a highly flexible process.

Single base mutations within its genome are key to varying susceptibility and resistance related behavior of HIV-1 in a host. Therefore, attempts to effectively detect these mutations hold key to identification of HIV-1 drug resistance.

The behavior of HIV-1 within host cell environment towards antiretroviral drugs is primarily driven by single base mutations within its genome. Such genetic changes result in enhanced drug resistance and therefore its timely and effective identification is of paramount clinical significance in anti HIV-1 management. The classical process of determining HIV-1 drug resistance mutations involve amplification of the *pol* gene by reverse transcription followed by Polymerase Chain Reaction (PCR) and nucleotide sequencing of the amplified region to detect the mutations that are associated with enhanced drug resistance and/or susceptibility.

In this study we demonstrate the utility of primer extension as a generic technology platform for identifying mutations within the HIV-1 genome. This method has potential to demonstrate faster turnaround time, superior quality of data and economy. Here we reported detection of M41L and M184V mutations within the reverse transcriptase gene by way of a multiplexed primer extension assay.

Material and Methods

Study population

The clinical panel used in this study included 75 HIV-1 sero-positive patients taking 1st line highly active antiretroviral therapy (HAART) and reported to have virological failure at the time of recruitment (Table 1). The drug resistance genotyping of this study panel was performed using the methods described by Acharya et al., [4]. The study was duly approved by SN Gene laboratory clinical research, institutional bio-safety and bio-ethics committee (Approval number DGL/2014/SQT2104). Written informed consent was obtained from all participants enrolled in this program.

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Variables	Summary, n=75
Age (yrs), median (IQR)	34 (25-43)
Gender, n (%):	
Male	41 (54.67)
Female	34 (45.33)
Median CD4T cell count, cells/µL (IQR)	149 (113-231)
Median Viral load, log10 copies/mL (IQR)	5.34 (4.19-5.82)
Risk Exposure, N (%)	
Heterosexual (%)	35 (46.67)
Bisexual (%)	26 (34.67)
MSM (%)	14 (18.67)
Other co-infections, n (%):	26 (34.67)
Treatment Regimen, N (%)	
AZT, 3TC, EFV	21 (28.00)
AZT, 3TC, NVP	17 (22.67)
TDF, 3TC, NVP	23 (30.67)
TDF, 3TC, EFV	14 (18.67)
IQR: Interquartile Range; MSM: Men who have Sex with Men; AZT: Zidovudine; 3TC: Lamivudine; EFV: Efavirenz; NVP: Nevirapine; TDF: Tenofovir	

Table 1: Demographic characteristics and laboratory results of the clinical study panel.

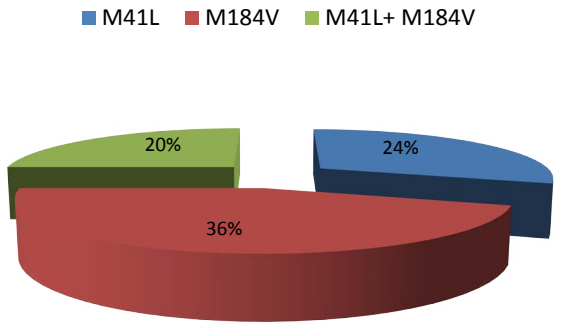

Figure 1: Frequency of M41L and M184V observed in the resource population.

Multiplex primer extension assay

The PCR amplicons generated using the protocol described by Acharya et al., [4] were purified using a QIAGEN PCR purification kit (QIAGEN, Germany) and directly used for the primer extension reaction. The reaction comprised of 5.0 µl of *SNaPshot* Multiplex Ready Reaction Mix (Life Technologies, USA), 0.4 pmol of PCR amplicon, 0.2 µM of each *SNaPshot* primers and nuclease free water to make up the volume to 10.0 µl. The thermal cycling condition of the reaction comprised of 25 cycles, each with a denaturation step at 96°C for 10 sec, an annealing step at 50°C for 05 sec and an elongation step at 60°C for 30 sec. Following primers were used in this study: 5'- AAA AAA AAA AAA AAA GCA TTA ACA GAA ATT TGT GAK GAA – 3' for detection of M41L and 5'- GACT GACT GACT GACT GACT GACT GACT GACT GACT GACT GACT CTA CAT ACA ART CAT CCA – 3' for detection of M184V.

Post extension treatment

One unit of Shrimp Alkaline Phosphatase (SAP) was added in the tube containing *SNaPshot* Multiplex assay reaction mix at the end of thermal cycling reaction and incubated at 37°C for 1 hour. The enzyme was then inactivated by incubating the reaction tube at 75°C for 15 minutes and immediately subjected to electrophoresis.

Electrophoretic resolution of the *SNaPshot* product

Nine µl of Hi-Di formamide was mixed with 0.5 µl of each primer extension (*SNaPshot*) product and GeneScan-120 LIZ size standard, denatured for 5 minutes at 95°C and subjected to automated capillary electrophoresis using a 3500 DX genetic analyzer (Life Technologies, USA) as per instruction of the manufacturer.

Molecular cloning of pcr fragments

RT-PCR fragments originating from HIV-1 with and without M41L and M184V mutation occurring together were cloned in pGEM®-T Easy Vector System II according to manufacturer's protocol and named pM41-M184 and p41L-184V respectively. pM41-M184 and p41L-184V DNA were isolated using a QIAGEN Plasmid Mini Kit [Qiagen, Germany] and estimated using a Qubit 3.0 fluorometer [Thermo Fisher Scientific, USA]. All dilutions of plasmid DNA were made in sterile distilled water.

Results

Study population

Details of the study population are summarized in Table 1.

Optimizing template quality for *SNaPshot* analysis

The template for *SNaPshot* assay developed in this study was essentially a Reverse Transcription (RT) PCR product generated by way of one step RT PCR protocol as described by Acharya et al. [4] Given the fact that minimal amount of template required to generate sufficient PCR products for *SNaPshot* or DNA sequencing is around 3 ng [5], we first attempted to perform the *SNaPshot* assay using unpurified RT PCR product. However, the results were inconsistent with regard to peak size and its position *vis a vis* size standard in different duplicate reactions prompting us to include PCR product purification as a mandatory step in the protocol.

Purified PCR products were used as a template to perform all subsequent *SNaPshot* assays which simultaneously detected two different SNPs under a multiplexed condition. Detection of the extended products was based on four different fluorescently labeled ddNTPs and the extension primers with oligonucleotide tails of differing lengths, thus controlling the concise length of the entire chromatogram to a defined number of bases. Both the mutations were easily identified on the basis of peak size and color. When a mutation was present, an alternative dideoxynucleotide triphosphate was incorporated, resulting in a different colored peak. A mutant peak was considered positive if it was three times above background noise in the wild-type sample. The mobility of extended primers in capillary electrophoresis was determined by their size, nucleotide composition, and dye.

Validation and sensitivity of the *SNaPshot* assay

Among 75 patients included in the study population and failing 1st line HAART, 18 (24.0%) M41L, 27 (36.0%) M184V and 15 (20.0%) with both mutations within reverse transcriptase gene of HIV-1 were observed (Figure 1). These results were in cent per cent concordance with those obtained by DNA sequencing based genotyping method as described by Acharya et al. [4,6].

To evaluate the sensitivity of *SNaPshot* assays, DNA from mutant plasmid [p41L-184V] harboring mutations of interest was diluted into wild-type plasmid [pM41-M184] in proportions of 10%, 5%, and 1%. Our experiments on plasmid model systems indicated the possibility of identifying at least 5% of mutated alleles in a background of wild-type DNA.

Discussion

The *SNaPshot* assay relies on accurate incorporation of a fluorescent

ddNTP at the 3'end of an extension primer that is designed to position one nucleotide behind the target single-nucleotide polymorphism (SNP) followed by fragment analysis [7]. This technology platform has found wide acceptance in different areas of clinical research. It has been employed for the quantitative detection of loss of heterozygosity in tumors [8], colorectal cancer [9], and mitochondrial DNA heteroplasmy [10]. In all such cases, the quantum of DNA applied was directly proportional to that of the amplified product and there was equal incorporation of ddNTPs in heterozygous samples also [7].

One of the first molecular typing assay on the primer extension platform was using Wheat streak mosaic virus (WSMV) as a model plant virus [11]. Later, the method was further extended for use in a multiplex format to evaluate a large panel of *Salmonella enterica* isolates. The result was found to be 100% specific and sensitive within the collection of isolates. It could be applied in less than 6 hours and had the potential to improve patient care and source tracing in cases of food poisoning [12].

Six common mycobacterial species namely *Mycobacteria tuberculosis, M. avium, M. intracellulare, M. chelonae, M. kansasii*, and *M. gordonae* were detected in a population of 468 mycobacterial clinical isolates using the primer extension technology. Out of this, 468 (99.15%) could be correctly identified by this assay thereby underlining the strong discriminatory strength for rapid and accurate identification of frequently encountered clinical mycobacterial species [13]. In the year 2009, Jakobsen and coworkers [14] demonstrated the utility of this technology for detecting mutations conferring drug resistance to HIV-1 reverse transcriptase inhibitors.

In this study, we identified 2 prominent and significant mutations within the reverse transcriptase gene of HIV-1 as targets for interrogation using the *SNaPshot* primer extension assay in a multiplexed format. These were M41L and M184V respectively.

Lamivudine (2, 3-dideoxy-3-thiacytidine; 3TC) is one of the most widely studied antiretroviral drug for HIV management because of its high level of clinical, biological and therapeutic superiority. The M184V mutation within HIV-1 genome and located in the reverse transcriptase gene is linked to enhanced resistance towards 3TC. Literature indicate that this genetic mutation results in alteration of a methionine residue at amino acid position 184 to valine and was extensively used for selecting viral cultures in laboratory for resistance towards didanosine (ddI) [15]. It was later found that the same mutation conferred high level of genetic resistance to the drug 3TC [16-22]. Furthermore, this mutation was found to be linked to almost all nucleoside analog RT inhibitors (NRTIs) [23-25]. M41L on the other hand, is a thymidine analogue mutation or TAM that is often found in association with T215Y. Both these mutations confer high level of resistance towards the drugs AZT, stavudine (d4T) and moderate level of resistance to the drugs didanosine (ddI), abacavir (ABC) and tenofovir (TDF). It is however interesting to note that virus with M41L + T215Y + M184V together mount moderate level resistance to AZT and d4T but reduced level of resistance towards the drug TDF [26]. In short, both M41L and M184V has rich history of imparting complex drug resistance traits to HIV-1 isolates and hence were identified as representative mutations for optimizing its detection by primer extension protocol in a multiplex format in this study.

The patients included in the study population were within the age group of 25 to 43 years recorded a mean CD4 count of 149 that was in line with their infection status. However, the strong presence of bisexual individuals at a high of 34.67% and MSM at 18.67% indicated a resounding gender preference pattern emerging within the HIV-1 infected population in the country that has the potential to indirectly dictate flow of resistant strains within the population.

We observed that minimal amount of carry over contamination that took place when unpurified RT PCR product of volume equivalent to 3 ng of DNA or more was sufficient to alter the consistency of the assay. It was therefore concluded that purification of template if it is a PCR product is advisable for a reproducible primer extension assay result.

The primer designed to detect M41L mutation comprised of a 5'-stretch of 15 bases followed by 24 specific ones out of which 1 was a degenerate base ('K') making it a 39 base long primer. On the other hand, the primer designed to detect M184L was 62 bases in length and comprised of a 5' stretch of 11 GACT repeats (44 bases) and 18 specific bases which included on degenerate base ('R'). The difference in primer lengths was sufficient to generate well spaced peaks on the electropherogram after incorporating the appropriate labeled dideoxynucleotide (Figure 2).

We observed acceptable limit of sensitivity of the assay which was 5% of the mutant allele in the backdrop of its wild type counterpart as was determined from a simulation experiment using recombinant & wild type plasmid clones. Given the fact that on several occasions the signal from mutant template population is camouflaged by wild type allele which occurs at an overwhelming frequency, this experiment was found to be useful in predicting the lower limit of detection of the mutant alleles in a heterogeneous population.

For the primer extension assay, a 1624 bp PCR amplicon was generated from the viral RNA by RT PCR and stored at -80^{0}C till further use. This PCR product covered the entire protease gene of the virus and more than first 300 amino acids of the reverse transcriptase gene which is hotspot region for the majority of HIV-1 mutations that confer resistance to clinically important antiretroviral drugs [27]. One of the main reason for optimizing the entire extension assay on this particular amplicon was to leave open the option to further expand the multiplexing capability of the assay by incorporating more number of mutations thereby creating useful 'panels' akin to those developed in oncology based assays [28]. Indian studies suggest that M184V is one of the common mutation in treated individuals [29]. The abundance of M184V within such patients was supported in our study also where we found that M184V occurred in around 56% of our study population out of which in 36%, it was found alone and in another 20%, in association with M41L. It is worthwhile to note that all 75 patients included in this study were under a definite treatment regimen as described in Table 1.

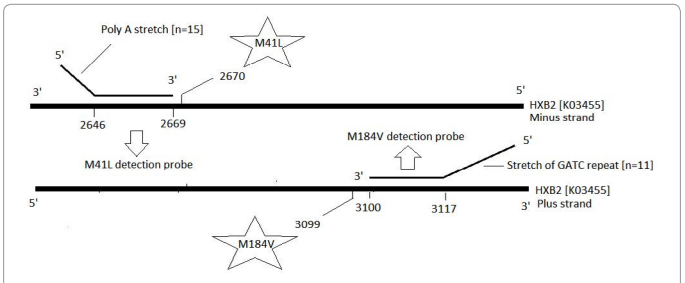

Figure 2: Diagram describing the *SNaPshot* primer location on the HIV-1 genome [HXB2, K03455]. The slanted line at the 5' end of the primers indicate non specific extension bases which is a 15 base poly A stretch of M41L probe and 44 base 'GATC' repeat, occurring 11 times in M184V probe. The location of target mutation M41L and M184V are shone within black stars and are positioned immediately adjacent to the last base at the 3' end of each probes. The hybridization location of M41L and M184V probes are on different strands of the HIV-1 genome and hence designed to extend in opposite directions.

Conclusion

This primer extension technology demonstrated in this study is flexible and amenable to multiplexing. Further, it is believed to be more sensitive compared to RT PCR and direct sequencing method. Needless to say, the results of primer extension and direct sequencing methods were definitely comparable.

Jakobsen et al., [14] reported occurrence of transmitted HIV drug resistance mutation or TDR in drug naïve HIV-1 patients at a higher frequency using HIV-*SNaPshot*, a primer extension protocol, compared to conventional RT PCR - nucleotide sequencing methodology. Such observations and findings from this study raises prospect of developing the primer extension *SNaPshot* method as a cost effective and efficient process in the days to come.

In this report, we demonstrated use of *SNaPshot* primer extension technology for the first time to detect M184V and M41L as representative mutations in Indian isolates of HIV-1 Subtype-C isolates.

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