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Drug Resistance Mutations to Protease and Reverse Transcriptase Inhibitors in Treatment Naive HIV-1 Clade C Infected Individuals from Mumbai, India

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ABSTRACT

The knowledge of drug resistance mutations in HIV patients is essential for clinicians to decide the optimal Antiretroviral Therapy (ART) combination. Interestingly, most studies reported from India have focused only on mutations in the Reverse Transcriptase (RT) region. Therefore, we carried out a pilot study to identify mutations in both the Protease (PR) and RT regions in treatment naive HIV seropositive patients from Mumbai. The PR and RT regions were successfully amplified and sequenced in fifteen samples using an automated DNA Sequencer. All specimens were identified as Clade C based on pol sequencing. Further, the drug resistance data was interpreted using the online Stanford HIV database. The most prominent mutations found in the RT region were T215 A/G/R (33.3%), Y181C (33.3%) and P225H (33.3%). It is noteworthy that although it is rare to observe major mutations in the PR region in treatment naive HIV patient samples, it was seen that M46L and I47M in >30% of the samples analyzed. Moreover, the homology of these sequences with HIV wild type sequence was verified using National Center for Biotechnology Information (NCBI) blast. Overall, many of the patient samples analyzed had mutations that impart resistance to Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Stavudine (D4T) and Efavirenz (EFV). Hence, despite the small sample size, the present findings highlight the fact that there may be higher levels of primary HIV drug resistance in Mumbai than previously reported.

Key words: HIV drug resistance, protease, reverse transcriptase, highly active antiretroviral therapy (HAART), anti retroviral therapy (ART)

INTRODUCTION

According to the AIDS Epidemic Update by the WHO (2008) it was estimated that an enormous 2.4 million people have been affected with HIV in India at the end of 2008 i.e., probably one in every eight people (Steinbrook, 2007). Of these, only an estimated 10-20% of infected people in India know that they have HIV infection (Steinbrook, 2007) and even fewer are those who have the privilege of well-timed drug resistance treatment and monitoring. The same is true in other developing countries like Nigeria (Olowosegun *et al.*, 2008), Cameroon (Alemnji *et al.*, 2006), Bangladesh (Khandoker *et al.*, 2006) wherein the very lack of knowledge of HIV status poses a big

challenge to control this disease. Fortunately, the easy accessibility and low cost of Highly Active Antiretroviral Therapy (HAART) since the year 2000 has dramatically decreased the AIDS (Acquired Immunodeficiency Syndrome) related mortality and improved survival of HIV patients who are aware of the disease (Palella *et al.*, 1998; Kumarasamy *et al.*, 2005). However, the emergence of drug resistant viruses has been an undesired outcome which requires timely monitoring. Especially, the first line therapy is of paramount importance in controlling viral replication and thus the primary mutations should be taken into consideration before prescribing first line regimen (O'Neil *et al.*, 2002). Several prospective controlled studies have shown that patients whose physicians have access to drug resistance data, particularly genotypic resistance data, respond better to therapy than control patients whose physicians do not have access to these assays (O'Neil *et al.*, 2002). This assists the physicians in the initial choice of treatment (Weidle *et al.*, 2003) and helps identify the genetic causes of treatment failure as a prerequisite in deciding the subsequent clinical course for better patient management (Kantor *et al.*, 2010).

HIV pathogenesis is influenced by various factors such as route of exposure, dose of the inoculum, genetic predisposition, age, environment, other opportunistic infections, virus variants etc (Oguntibeju *et al.*, 2007). Further, HIV-1 clade C infected persons comprise more than 56% of the infections worldwide and more than 98% of the infections in the Indian subcontinent (Robertson *et al.*, 2000). Surprisingly, existing sequence data and interpretation algorithms are instead focused on HIV-1 clade B. Compared to the extensive information available on the B subtypes, there is hardly enough statistics on the predominant non-B subtypes to identify significant correlations and develop a sequence database. Therefore, extensive surveillance efforts are necessary to control the widespread dissemination of the clade C viruses.

The assay for antiretroviral response has become an indispensable diagnostic tool to increase the overall efficiency of treatment and management of HIV affected patients. The genotypic technique by gene sequencing remains the 'gold standard' for resistance testing due to the ability to detect mutations in variants that are present in low quantities as that can potentially cause resistance and also detect mutations that are responsible for selective drug pressure (Shafer, 2002). However, in India the laboratories having the facilities for genotyping are very few (Robertson *et al.*, 2000). Moreover, the enormous cost of this assay makes patients shy away from it. Drug resistance testing has not been an integral part of routine treatment monitoring of patients receiving ARV drugs or as a prerequisite to initiate ARV treatment. Furthermore, there is a paucity of information regarding the presence of primary drug resistance to protease inhibitors in Indian literature (Shankarkumar *et al.*, 2009). Especially, the studies on primary drug resistance mutations reported from Mumbai have mainly focused only on the reverse transcriptase region of the pol gene. Therefore, this study has analyzed primary drug resistance mutations in both protease and reverse transcriptase regions, in treatment naive HIV seropositive individuals from Mumbai, India. This is a report on the preliminary findings on drug resistance mutations in clade C individuals which may contribute to the HIV mutations data at a global level.

MATERIALS AND METHODS

Sample collection: The patients attending the clinics of Jaslok Hospital and Research Centre and other hospitals in Mumbai from the year 2008-2010 were enrolled for this study. The Scientific Advisory committee and Ethics committee of Jaslok Hospital and Research Centre had approved this study. Fifty naive HIV seropositive patients and 10 control individuals were enrolled with their informed consent. Five ml of blood was collected in EDTA BD Vacutainer® tubes and RNA isolation was immediately carried out using the Trizol® reagent (Invitrogen, USA).

HIV RNA PCR: HIV RT-PCR was performed using the QIAGEN® OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). The upstream primer SK462 (5'-TGC TAT GTC AGT TCC CCT TGG TTC TCT-3') and downstream primer SK438 (5'- AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT-3') were utilized to amplify the HIV gag region. The cycling conditions were as follows: 50°C for 30 min, 94°C for 2 min, 40 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 1 min and final extension at 72°C for 5 min. The 142 bp amplicons obtained were loaded an 8% polyacrylamide gel and electrophoresis was carried out under non-denaturing conditions and visualized by silver staining.

HIV real time PCR: HIV RNA real time PCR was performed using RoboGene Human Immunodeficiency Virus (HIV-1) quantification kit (aj ROBOSCREEN, Germany). The 200 ng of Trizol® extracted RNA was added to the Master mix containing 2 x universal master mix+primer (200 nM) + probe (200 nM) + 1x RT enzyme (SuperScript™ III Platinum). For each experiment, a non template control (NTC) and a negative control (HIV seronegative individual's RNA confirmed by PCR) was run. The external control RNA was HIV-1 RNA (aj ROBOSCREEN, Germany) at various dilutions. The samples were transferred into a 96 well real time PCR plate and the plate was kept into the real-time PCR instrument (ABI 7700, Foster City, USA). The thermal conditions were set at 60°C for 45 min for initial incubation of reverse transcription, hold on 95°C for 2 min and then proceeding with 45 cycles of melting at 95°C for 15 sec and annealing at 57°C for 1 min. Viral copies were calculated from the Ct value using a proper standard graph and quality control measures were taken at every step.

Genotypic assay for detection of HIV drug resistance: The sequencing of the protease and reverse transcriptase genes was performed as described in the past by Balakrishnan *et al.* (2005) with a few modifications.

First round PCR: The RNA extracted was used in a Reverse transcriptase PCR using the QIAGEN® OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). The primers used were upstream PR1 (5' - ACC AGA GCC AAC AGC CCC ACC A-3') and downstream PR2 (5'- CTT TTG GGC CAT CCA TTC CTG GC-3') for protease and upstream RT1 (5'-TAG GAC CTA CAC CTG TCA ACA TA-3') and downstream RT6 (5'-TAG GCT GTA CTG TCC ATT TAT CAG G-3') for reverse transcriptase genes. The same cycling conditions for both protease and reverse transcriptase were used in the first round PCR which was set up at 50°C for 30 min, 94°C for 2 min, 40 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 1 min and 72°C for 5 min.

Second round PCR: The 2 μL of the first PCR products was used as template for the second round PCR. For the protease gene, the primers used were upstream PR3 (5' -GAA GCA GGA GCC GAT AGA CAA GG-3') and downstream PR2 (5'- CTT TTG GGC CAT CCA TTC CTG GC-3') and the PCR conditions were initial denaturation at 95°C for 1 min, 25 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, extension 72°C for 45 sec and final extension at 72°C for 7 min. For the reverse transcriptase gene, the primers used were upstream R3 (5'- TAG GCT GTA CTG TCC ATT TAT CAG G-3') and downstream RT6 (5'-TAG GCT GTA CTG TCC ATT TAT CAG G-3') with the PCR conditions set up at initial denaturation at 95°C for 1 min with 25 cycles of denaturation at 95°C for 15 sec, annealing 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min.

The amplicons obtained for both protease and reverse transcriptase genes were analyzed on a 2% agarose gels stained with ethidium bromide (20 mg mL^{-1}) and observed under UV light. The purified PCR product was subjected to DNA sequencing using Big Dye Terminator (Applied Biosystems, USA) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Analysis using HIV stanford database: The sequences obtained were analyzed for homology with HIV protease and reverse transcriptase genes using NCBI BLAST search and HIV clade detection was done using the online NCBI Viral Genotyping tool. Further, the online HIV Stanford Database (http:// hivdb.stanford.edu) was utilized for genotypic drug resistance analysis and all sequences were defined as compared with clade B as per the database rules.

RESULTS

Preliminary observations (Table 1) of the HIV seropositive naive patients enrolled for the study regarding gender and age revealed that 76% were males and their ages ranged from 7 to 55 years with a median age of 30 years. HIV seropositivity was confirmed by RT-PCR of gag gene expression for all fifty samples. Out of the 50 HIV seropositive patients, 18 samples had an undetectable viral load i.e. plasma HIV-1 RNA viral load = 1000 copies mL^{-1} and thus could not be sequenced. The remaining samples were within a range of 1.4×10^3 to 2.8×10^6 viral copies mL^{-1} and had a median value of 3.6×10^5 viral copies mL^{-1}.

HIV Protease and reverse transcriptase regions of the pol gene were successfully amplified and sequenced in fifteen samples. RT-PCR for the reverse transcriptase and protease regions have been shown in Fig. 1 and 2, respectively. Further, clade identification was done using the NCBI viral mutations (Table 2) at the following positions: V32I (1 out of 15); M46L (5 out of 15); I47M (8 out of 15); I50V (1 out of 15); V82A/D/G (6 out of 15); N88S (3 out of 15). This study further showed that M46L, I47M and V82A/D/G were most prominent mutations found in almost more than 30% of patients analysed for this study and this needs further investigation with more number of samples. These PR mutations impart resistance to drugs like Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV) etc. Also, minor protease mutations/polymorphisms in ten of the fifteen samples studied were seen at the following positions: L10I (1 out of 15); V11F (1 out of 15); L23H (2 out of 15); K43I/R (2 out of 15); A71D (1 out of 15); G73A (2 out of 15); T74A/P/S (8 out of 15); N83D (8 out of 15). As far as Reverse transcriptase gene mutations are concerned, Nucleotide

Table 1: Preliminary observations of HIV-1 infected individuals enrolled for the study

Preliminary observations	Patients (n = 50)
Gender	
Males (%)	76
Females (%)	24
Age	
Range (years)	7-55
Median (years)	30
Viral load	
Number of detectable samples i.e., >1000 copies mL^{-1}	32*
Number of undetectable samples i.e., <1000 copies mL^{-1}	18
Range of viral load of detectable samples (copies mL^{-1})	1.4×10^3 to 2.8×10^6
Median of viral load of detectable samples (copies mL^{-1})	3.6×10^5

*Out of 32 samples which had detectable HIV viral loads, 15 samples were successfully sequenced
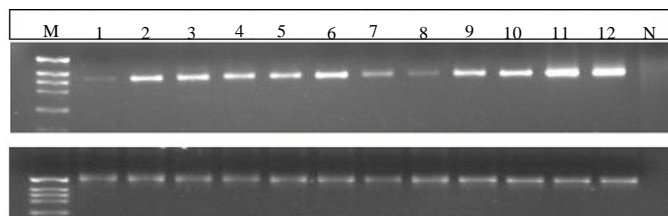
Fig. 1: A region in the HIV reverse transcriptase obtained by 1 step RT-PCR and subsequent nested PCR (above) and the amplification of beta-Actin gene (below), respectively
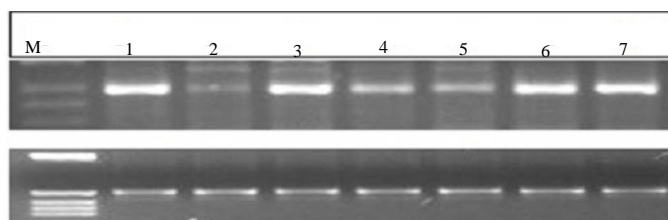

Fig. 2: A region in the HIV protease obtained by 1 step RT-PCR and subsequent nested PCR (above) and the amplification of beta-Actin gene (below), respectively

Table 2: Major protease drug resistance mutations obtained in indian HIV seropositive naive patients

Mutation	No. of patients	%	Reduced sensitivity to drugs
V32I	1/15	7	All PI, except Saquinavir (SQV)
I47M	8/15	53	Reduced susceptibility to FPV, IDV, LPV, TPV, DRV. Highly unusual mutation
V82A/D/G	6/15	40	Reduce susceptibility to IDV, LPV with other mutations NFV ATV, FPV. V82DG highly unusual mutation.
N88S	3/15	22	High resistance to NFV, ATV, increased susceptibility to FPV
I50V	1/15	7	Causes intermediate resistance to FPV
M46L	5/15	33.35	Resistance to IDV, NFV, FPV, LPV, ATV

FPV: Fosamprenavir, ATV: Atazanavir, IDN: Indinavir, LPV: Lopinavir, TPV: Tiprinavir, DRV: Duranavir, NFV: Nelfinavir, SQU: Saquinavir

Reverse Transcriptase Inhibitors (NRTIs) mutations at positions M41L (1 out of 15); D67N (1 out of 15); M184V (2 out of 15); T215A/G/R (5 out of 15); K219E/R (3 out of 15), L210V (1 out of 15) were seen as shown in Table 3. The most prominent among these was T215A/G/R as seen in 33.3% of the study cohort. Mutations associated with Non Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) were also observed in this study at positions: Y181C (5 out of 15); Y188L (1 out of 15); G190A (1 out of 15); H221F/P (4 out of 15); P225H (3 out of 15). Therefore, Y181C (33.3%), H221F/P (26.6%) and P225H (20%) were the prominent mutations seen in the study cohort as shown in Table 4. Overall, many of the patients whose samples were sequenced for RT were resistant to Stavudine (D4T) and Efavirenz (EFV). This information is particularly useful in a country wherein resource limited settings and financial constraints limit the access to genotypic drug resistance data, thereby further investigations on the prevalence of these mutations in the

Table 3: NRTI mutations found in present study in Indian HIV seropositive naive patients

Mutation	No. of patients	%	Resistant to drug
T215A/R/G	5/15	33.3	AZT, D4T, ABC, DDI and TDF and is highly unusual.
M41L	1/15	7	Usually occurs with T215G High level resistance to AZT, D4T, ABC and TDF
M184V	2/15	14	High resistance to 3TC, FTC, increased susceptibility to AZT, TDF and D4T
D67N	1/15	7	Low resistance to NRTIs, susceptible to 3TC, FTC.
K219E/R	3/15	20	Decreased susceptibility to AZT and D4T
L210V	1/15	7	Usually occurs with the mutations M41L and T215Y and is highly unusual

AZT: Zidovudine, D4T: Stavudine, ABC: Abacavir, DDI: Didanosine, TDF: Tenafovir, FTC: Emtricitabine, 3TC: Lamivudine

Table 4: NNRTI Mutations found in our study in Indian HIV seropositive naive patients

Mutation	No. of patients	%	Resistant to drug
Y181L	1/15	7	High level resistance to NVP and EFV Low resistance to ETR
Y181C	5/15	33.3	Cause high level resistance to ETR, NVP/PLV, Low resistance to EFV
H221F/P	4/15	26.6	It is uncommon NNRTI associated mutation
P225H	3/15	20	Increased EFV resistant when in presence of P225LQR
G190A	1/15	7	Causes high level resistance to NVP and EFV and increased DLV susceptibility

NVP: Nevirapine, ETR: Etravivine, DLV: Delavidine, EFV: Efavirenz

Indian HIV gene pool can lead to the avoidance of these drugs for the treatment of HIV clade C patients of Indian origin.

DISCUSSION

According to the UNAIDS (2010) findings HIV in India is more prevalent in males than females which is concordant with the current findings. The same was true in a research study conducted in Malaysia consisting of 693 newly diagnosed HIV patients (Nissapatorn *et al.*, 2007). Conversely, HIV seems to be equally prevalent in males and females in other developing countries such as Nigeria (Akhigbe *et al.*, 2010) and more prevalent in females in other Nigerian studies (Nwachukwu and Orji, 2008; Amegor *et al.*, 2009). Further, the accurate diagnosis of this disease largely relies on PCR techniques rather than serological assays (Jamehdar *et al.*, 2007), hence we confirmed the HIV seropositive status of all the patients using RT-PCR. Furthermore, HIV drug resistance assays are inseparable components of effective clinical management of individuals commencing or undergoing ART. The events of early HIV infection, mainly the saturation of the target cells, influences the transmitted drug resistant viruses to become well established in reservoirs that maintain long term resistance (Brenner *et al.*, 2002; Delaugerre *et al.*, 2004; Ghosn *et al.*, 2006). Also, the replicative fitness of the viruses can be reduced with the correct ART combination (Gandhi *et al.*, 2003). Therefore, the initial knowledge of the resistance status in an HIV seropositive individual commencing antiretroviral therapy is extremely vital. The International AIDS Society (IAS), USA, also recommends genotypic testing before commencing ART as this will detect mutations that may not be revealed in phenotypic assays and identify the subtype (Hirsch *et al.*, 2008). In the present investigations, 15 samples could be screened for protease inhibitor resistance mutations of which many major and minor mutations were detected. It is noteworthy that although it is rare to observe major mutations in the PR region in treatment naive HIV patient samples, we came across many major mutations in the samples analyzed. Confirmation of the homology of these sequences with HIV wild type sequence was done using NCBI blast. The plausible explanation for observing many PR mutations is that the infrequent occurrence of major PR mutations makes many researchers overlook PR mutation analysis, which is also a tricky task.

It was observed that I47M (substrate cleft position), a rare mutation, was present in more than 50% of samples analyzed. Interestingly, Mousavi *et al.* (2010), have recently reported I47M in a study from Iran. Isoleucine (I) at position 47 is typically mutated to valine(V) or Alanine(A) leading to decreased susceptibility to Fosamprenavir (FPV), Atazanavir (ATV), Indinavir (IDV), Lopinavir (LPV), Tipranavir (TPV) and Darunavir (DRV). Additionally, two most common mutations detected were M46L (flap position) and V82A/D/G (substrate cleft position). The former mutation is responsible for reducing susceptibility to almost all the protease inhibitors namely IDV, Nelfinavir(NFV), FPV, LPV, ATV, TPV and possibly Saquinavir (SQV) and DRV. The latter mutation has been reported as 10% of all primary mutations in a recent study from western India by Sachdeva *et al.* (2005) and results in contraindication to IDV and LPV. Further, mutation at position 88 (interior enzyme position) was observed in 22% of samples analyzed causing high level resistance to ATV and NFV. Mutations at position 82 and 88 are generally co-existent and result in contraindication to many PIs particularly NFV. Other major mutations in the protease gene were seen in low frequency (6%) at positions V32I and I50V which result in high level resistance to FPV. Besides, minor mutations/polymorphisms in the protease region were also observed in high frequencies the most common being at positions 74 (53%) and 83 (53%). These polymorphisms may have a negligible contribution to drug resistance. Nevertheless, physicians have to take into account these accessory mutations before making treatment decisions as they may be play a role in increasing viral fitness in association with major mutations (Alexander *et al.*, 2001; Cane *et al.*, 2001; Cerquerira *et al.*, 2004; Escoto-Degadillo *et al.*, 2005; Han *et al.*, 2007; Paraschiv *et al.*, 2007)

Studies from Southern India on ART naive individuals show absence of any primary NRTI and NNRTI drug resistance mutations (Balakrishnan *et al.*, 2005; Soundararajan *et al.*, 2007). In contrast, 6 NRTI and 5 NNRTI mutations were noticed in most samples that were screened for reverse transcriptase region. This is in agreement with earlier studies from Mumbai, India, which report prevalence of primary drug resistance (Deshpande *et al.*, 2004). The resistance to Nucleotide Reverse Transcriptase Inhibitors were most frequent (33.3%) at position 215 known as Thymidine Analog Mutation (TAMs) which was seen alone or in combination with K219E. Moreover, TAMs are common in low-income countries and decrease susceptibility to Nevirapine (NVP) and Stavudine (D4T). The next most prevalent mutation was M184V seen in 14% of the samples analyzed. This is the most commonly occurring NRTI mutation and confers high level resistance to lamivudine (3TC) and emtricitabine (FTC). Thus, a higher prevalence of T215Y than M184V was seen while a study from Northern India among ART naive individuals have reported higher prevalence of M184V (Hira *et al.*, 2004). This may be due to several factors such as differences in sample size, geographical distribution, mode of transmission etc. Further, a single case of 4 NRTIs namely M41L, D67N, T215A, M184V along with M46V protease inhibitor mutation was observed which may result in high/intermediate level resistance to many drugs except for NVP. Although, these are isolated cases among ART naive individuals, they highlight the fact that genotyping for drug resistance mutations may be a crucial factor in commencing antiretroviral therapy. Other NRTIs include L210V (7%) and K219E (15%). L210V which was recently reported in naive subjects from Italy (Monno *et al.*, 2009) and its more common form, L210W confers resistance to each of the NRTIs except for 3TC and FTC.

Regarding the NNRTIs, the most common mutation observed was Y181C (33.3%). The same has been true for HIV-1 infected pregnant women from south India after single dose NVP (Rajesh *et al.*, 2010). Y181C is primarily responsible for resistance to NVP and DLV and low-level resistance to EFV. Additionally, Y was substituted by L at position 181 in one case which is an

unusual mutation at this position. Subsequently, the next most prominent mutation was as position 221(26.6%) which is concordant with a recent study in patients from Mumbai undergoing NRTI plus NNRTI (Deshpande *et al.*, 2007). Besides, P225H was detected in 20% of the cases. This being a secondary mutation confers resistance to EFV when in combination with other mutations like K103N, Y188L or G190A (Bacheler *et al.*, 2001). Likewise, G190A was seen in one individual in combination with P225H. Substitution of A instead of G has also been recently observed by Kandathil *et al.* (2009) in clade C infected individuals in India. By and large, the NNRTIs observed generally impart EFV resistance.

Overall, despite some limitations in this study like small sample size, unknown routes of transmission etc., the findings well correlate with the fact that clade C viruses predominate in India (Robertson *et al.*, 2000). Furthermore, the prevalence of drug resistance mutations in HIV-1 infected untreated patients in India was hitherto thought to be low (Pachamuthu *et al.*, 2006; Chaturbhuj *et al.*, 2010). Most notably, the present results suggest that these primary drug resistance mutations need to be thoroughly investigated and large-scale studies are required to evaluate the transmission of HIV drug resistance in Mumbai and other parts of India. Genotypic drug resistance data can give clinicians a true picture of drug resistance and susceptibility and ensure the commencement of the most suitable ART combination for the management of this disease.

CONCLUSIONS

The current findings draw attention to the fact that there may be high levels of primary HIV drug resistance in Mumbai. Further, from the HIV drug resistance mutations analyzed in our cohort, the most prominent resistant drugs were: Indinavir (IDV), Lopinavir (LPV), Nelfinavir(NFV), Stavudine (D4T) and Efavirenz (EFV). Simply put, the genotypic assay for HIV drug resistance is imperative to initiate and monitor the progress of ART. Further, continued efforts in HIV molecular epidemiology is essential which will not only aid in effective patient management but also enhance our understanding on the clade C mutations prevalent in the Indian HIV gene pool.

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