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Screening of Drug Resistance-associated Mutations in Polymerase Gene of HIV-1 Subtype C.

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ABSTRACT

This study aimed to evaluate the mutation of drug resistance in the pol gene of Human immunodeficiency virus (HIV)-1 strain isolated from Indian subtype-C antiretroviral (ARV) treatment-naive patients. Our study was designed to obtain, mutation data on the drug-resistant variants in a treatment-naive Indian subtype C population and to detect Low-level Drug-resistant HIV Variants. A total of 93 HIV-1 strains isolated from treatment-naive patients were included in this study. Resistance genotyping for the pol gene was performed using nested Polymerase chain reaction (PCR) and Deoxyribonucleic acid (DNA) sequencing. The sequences were aligned (ClustalW) and Drug resistance (DR) pattern was analyzed using the Stanford HIV-DR database and the IAS-USA. As a result, we standardized a specific and sensitive HIV genotyping PCR-based technique to detect drug resistance associated mutation in polymerase gene which specifically codes for protease and reverse transcriptase enzyme. In this study, we developed a cost effective, sensitive genotyping system for HIV drug resistance specifically for Indian subtype C in the Protease (PR) and Reverse Transcriptase (RT) regions of HIV pol gene.

Keywords: HIV, Drug Resistance mutation, PCR, Protease and Reverse Transcriptase, *pol* gene, Indian HIV-1 subtype C.

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INTRODUCTION

Human immunodeficiency virus (HIV) is one of the retroviruses, commonly it have an inherently high mutation rate, caused by copy errors during replication and the lack of a proof-reading function to correct those errors. Human immunodeficiency virus type 1 (HIV-1) subtype C viruses are associated with nearly half of worldwide HIV-1 infections and are most predominant in India. HIV Wild type viruses are the most susceptible to antiretroviral (ARV) drugs. The development of resistance to antiretroviral medications is the main cause for treatment failure of HIV-infected individuals [1]. Antiviral drug resistance is defined by the presence of viral mutations that reduce drug susceptibility compared with the susceptibility of wild-type viruses.

The genome of HIV consists of three major genes; *Group specific antigen* (*gag), DNA polymerases (pol) and Envelope (env).* In this study we focus only on *pol* gene. The *Pol* gene is one of the main retroviral genes, located in HIV, which has the four different proteins namely - Reverse transcriptase (RT), Protease (PR), RNAse H and Integrase (IN). The role of RT protein- is to copy the virus RNA into double stranded DNA by replication process, PR- cuts the proteins into segments, RNAse H- breaks down the retroviral genome during infection of a cell, IN- integrates the DNA of HIV's genome into the host DNA [2,3].

Most genotypic assays involve sequencing of the RT and PR regions of the *pol* gene to detect mutations that are known to confer drug resistance. Two main commercial HIV genotyping assays are available to monitor drug resistance, ViroSeq (Abbott) and TRUGENE (Siemens, Washington DC) [4-7]. The high cost associated with commercial assays hinders their use in resource-limited settings.

In this study, we adopted and evaluated a low-cost, specific and sensitive nested Polymerase chain reaction (PCR) and sequencing method to detect the low level drug resistance mutations in pol gene which specifically codes for protease and reverse transcriptase enzyme in a treatment-naive Indian subtype C population. These assays will be a useful tool in studies examining the prevalence of specific mutations in different populations and determining the impact of mutations on therapy outcomes.

METHODOLOGY AND METHODS

Clinical Specimens & Viral RNA Extraction

In this study we collected the HIV-1 clinical isolates from antiretroviral drug-naive patients of Indian subtype C in different distinct sites from Andhra Pradesh. A total of 93 patients with known drug resistance genotypes were recruited into the study. Viral RNA (vRNA) was extracted from 140µl of plasma samples from HIV-1-infected individuals using QIAmp® viral RNA Mini Kit (QIAGEN, Germany).

Reverse Transcription (cDNA synthesis)

The purified RNA was used for cDNA synthesis using Invitrogen Superscript III cDNA synthesis Kit (USA). Oligo dT and Random Hexamer primers are used to convert the RNA into first strand cDNA. PCR were performed with 10µl reaction mixtures (RNA, primer and dNTPs) in a 0.2ml. Denaturation step was carried out at 65ºc for 5 minutes, and placed on ice for 1 minute. Then 10µl of cDNA synthesis mix was added (10x RT buffer, 25mm Mgcl2, 0.1 M DTT, RNase OUT and Superscript III RT) and incubated at 50 ºc for 50 minutes. The reactions were terminated at 85º C for 5 minutes and finally 1µl of RNase H was added to each tube and incubated for 20 minutes at 37º C. (to remove the excess RNA). The cDNA were used for HIV drug resistance assay using PCR techniques.

PCR for Pol gene Of HIV-1 Subtype C

In this study, we optimize a specific and sensitive PCR-based strategies to identify subtype C-viruses**.** The RT region was amplified by using nested PCR and the PR region was amplified by using hot start PCR. We designed the Drug Resistance primer for both RT and PR region of the *pol* gene in HIV-1 subtype C (Table 1). The reaction conditions for nested and hot start PCR were shown in Table-2. The RT region amplicon size was a 665-bp and PR region amplicon size was a 459bp. The PCR products were visualized on a 2% agarose gel with ethidiumbromide staining. The amplified products were sequenced in Mega BACE 1000 system using Sanger's

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dideoxy chain termination method [8]. The sequences were aligned in NCBI database and the mutations were analyzed with Stanford University HIV Drug Resistance Database.

Table 1: Primer Sequences of RT & PR Gene for Drug Resistance Mutations.

S.NO	*Pol* Gene	Primer sequences for Drug Resistance Mutations	Basepair (bp) Size
1	RT gene	**Nested Primers-** **First Round** HIVRT F 5'-TTG ACT CAG ATT GGT TGC ACT TTA A-3' HIVRT Rev 5'- TGG AGG TTC TTT CTG ATG TTT G-3' **Second Round** HIVRT2 F 5'- GAC TTA GAA ATA GGG CAG CAT AGA A-3' HIVRT2 Rev 5'- GGT TTC TGC TCC TAC TAT GGG TTC T-3'	665bp
2	PR gene	HIVDPr F- 5'-AGC AGG AGC CGA TAG ACA AGG AA-3' HIVDPr R- 5'-TGG TAC AGT CTC AAT AGG GCT AAT G-3'	459bp

Note: *Pol* Gene- Polymerases (Pol), RT gene- Reverse transcriptase (RT), PR gene- Protease (PR)

Table 2: PCR Programme Conditions for RT & PR Genes.

S.NO	*POL* GENE	PCR CONDITIONS
1	RT gene	The **Nested PCR** conditions were as follows: **First Round**- 94˚c for 13min followed by 25 cycles of 94˚c for 45 sec, 55˚c for1 min and 68˚c for 2 min. Final extension step at 68˚c for 7 min. **Second Round (Nested)** - Same as first round, but cyclic condition are 35.
2	PR gene	**Hot start PCR**: The PCR conditions were as follows: 94˚c for 13min followed by 35 cycles of 94˚c for 30 sec, 60˚c for1 min and 68˚c for 1min. Final extension step at 68˚c for 7 min.

Note: *Pol* Gene- Polymerases (Pol), RT gene- Reverse transcriptase (RT), PR gene- Protease (PR)

RESULTS

The presence of the target mutations was confirmed by Stanford University HIV Drug Resistance Database. Major HIV drug-resistance mutations were isolated from two of 93 patients. No major PI mutations were observed in our study (Table3) but some accessory minor PI mutations L63T, I64L, H69K, V82I, and I93L were seen. One patient (85.1%) had RT mutation, F77P, K219Q that imparts resistance to NRTIs and NNRTI resistance to V90I, Y188F, zidovudine (AZT) and stavudine (D4T). (Table 4). Accessory minor RT mutations are E79G, L80KMRT, N81CFSY, K82HLPR, R83H, T84R, Q85S, D86S, F87S, E89R, G93N, S117T, L120F, D121N, K122E, I135K, S162Y, T165I, K173A, Q174K, I178L, T200A, Q207E, and R211K. These mutations facilitate dual resistance to AZT and Stavudine (D4T) in association with K70R or T215Y/F.

Table 3: Protease(PR) Drug Resistance Interpretation:

Protease(PR) Drug Resistance Interpretation:	
PI Major Resistance Mutations:	None
PI Minor Resistance Mutations:	None
Other mutation	**L63T, I64L, H69K, V82I, I93L**
Protease Inhibitors(PI)	
atazanavir/r(ATV/r)	Susceptible
darunavir/r(DRV/r)	Susceptible
fosamprenavir/r(FPV/r)	Susceptible
indinavir/r(IDV/r)	Susceptible
lopinavir/r(LPV/r)	Susceptible
nelfinavir(NFV)	Susceptible
saquinavir/r(SQV/r)	Susceptible
tipranavir/r(TPV/r)	Susceptible

Note: Protease Inhibitors (PI)

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Table 4: Reverse transcriptase (RT), Drug Resistance Interpretation:

Reverse transcriptase (RT), Drug Resistance Interpretation:	
NRTI Resistance Mutations:	**F77P, K219Q**
NNRTI Resistance Mutations	**V90I, Y188F**
Other Mutations:	**E79G, L80KMRT, N81CFSY, K82HLPR, R83H, T84R, Q85S, D86S, F87S, E89R, G93N, S117T, L120F, D121N, K122E, I135K, S162Y, T165I, K173A, Q174K, I178L, T200A, Q207E, and R211K**
Nucleoside RTI	
lamivudine (3TC)	Susceptible
abacavir (ABC)	Susceptible
zidovudine (AZT)	Low-level resistance
stavudine (D4T)	Potential low-level resistance
didanosine (DDI)	Susceptible
emtricitabine (FTC)	Susceptible
tenofovir (TDF)	Susceptible
Non-Nucleoside RTI	
delavirdine (DLV)	Susceptible
efavirenz (EFV)	Susceptible
etravirine (ETR)	Susceptible
nevirapine (NVP)	Susceptible

Note: NRTI- Nucleoside Reverse transcriptase Inhibitors, NNRTI – Non-Nucleoside Reverse transcriptase Inhibitors.

DISCUSSION

HIV infection in humans is now pandemic. As of July 2014,the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization(WHO) estimated that more than 35 million people lived with the AIDS worldwide, and it killed an estimated 1.5million people in 2013. In Asia and the Pacific, nearly 350,000 people became newly infected in 2013, bringing the total number of people living with HIV there to 4.8 million [9]. India has a third largest number of HIV infections in the world.

Hardly few studies on drug resistance in antiretroviral therapy (ART)- naive patients carried out in southern part and south western part of India and it disclosed no primary NRTI and NNRTI drug resistance mutations [10,11]. Nevertheless 1.7% prevalence of primary drug resistance were described from Mumbai [12,13]. The study of ART-naive patients from Chandigarh, in the northern part of India, revealed a high prevalence of M184 V/I (32%) and K70R (78.3%) and a low prevalence of T215 Y/F (1.67%) [14].

Latterly, HIV-1-infected ART-naive patients from western India were reported 10% of primary resistance mutation V82A in PR gene and; M41L, D67N, M184V, and A98G in RT gene [15,16]. Mutations which were reported in the literature- K70R, T215F/Y for ZDV treatment [17], N88S in the PR and V118I in the RT, R8Q/K- substrate cleft, L10I/F/V/R-Accessory, M46I-Enzyme flap M46I [18]. Julius M,et al.,2011 analyzed the mutation of M46I in PR region of the pol gene and G190V of RT gene [19].

Mutations in D67E, D67G, and T69D were detected in three women while T215Y and M46I were detected in two men. Several polymorphisms, such as K20R, M36I, and I93L, common in HIV-1 subtype C viruses and associated with drug resistance site [20]. In 2013, Azam M et al., studied the drug resistance-associated mutations in HIV-1 subtype C PR gene, were observed major mutation at D30 N and M46I positions. Minor mutations were observed in PR gene, of which 14 distinct amino acids changes were linked to partial DR such as G16E, K20R, M36I, D60E, I62V, L63P, I64M, H69K, T74A/S, V77I, V82I, I85V, L89M, and I93L [21].

The present study was undertaken to analyze the potential emergence of drug resistant HIV-1 sub type-C in subjects. When analyzing the rate of accumulation of mutations conferring drug resistance over time for each major ARV class, subtype C viruses apparently acquired a lower number of mutations than subtype B for PI and NRTI, but not for NNRTI. Overall, mutation were observed in the PR gene region was less conserved than the RT gene region. No major mutations conferring resistance to PIs were seen, but few

of the strains had two or more minor mutations associated with PI resistance; only some of strains (mainly subtype C strains) had some minor mutations. Amino acid substitutions associated with PI resistance have been reported as natural variants in treatment-naive patients (L 63T, I64L, H69K, V82I and I93L).

CONCLUSION

In this study, we developed a cost effective, sensitive genotyping system for HIV drug resistance specifically for Indian subtype C in the PR and RT regions of HIV pol gene. Our study reveals that the HIV-1 drug resistance testing will facilitate the decision making for the appropriate management of HIV infected patients. We also state that the genotyping resistance testing is recommended to help guide the choice of new drugs for pregnant women and also for the patients non responsive to treatment.

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