ORIGINAL RESEARCH ARTICLE

Detection of Latent HIV-1 Infection and Drug Resistant Mutation Testing in Nepal: HIV-1 *env* V3 DNA Sequence and *RT* Gene (M184V) Mutation

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Abstract

HIV-1 resistance to antiretroviral therapy (ART) is a crucial issue, despite various effective drugs are available for the treatment. Although the viral RNA is suppressed below the detection limit (<50 copies/ml) with the use of potent antiviral drugs, the mutation can be archived in the cellular reservoir as proviral DNA. The detection of proviral DNA and mutation screening in HIV 1 RNA for genotypic resistance is the sole basis for monitoring the effectiveness of ART. Our study aim to access the extent of latent HIV infections by detecting *env* V3 DNA and also testing of M184V (meth184val; ATG - GTG substitution at 184th codon) specific mutations in HIV-1 *RT* gene to monitor the effectiveness of ART. The HIV-1 *env* V3 DNA sequence was amplified using multiple upstream and downstream primes to show the latent HIV infections, whereas polymerase chain reaction-restriction fragment digestion assay (PCR-RFDA) was used for testing M184V mutation in HIV-1 *RT* gene. In the study, out of 15 HIV infected patient blood samples, 12 shows amplification of env V3 DNA, confirming the latent HIV infections while 3 were negative for *env* V3 DNA. HIV-1 *RT* gene tested for M184V mutation in all 15 samples showed wild type after analysis using PCR-RFDA. After digestion with CviAII, three bands were observed in wild type whereas in mutant only two bands. Although the study shows negative for the M184V resistance mutation, screening of various panels of drug resistance mutations should be performed in recently infected HIV-1 patients for planning the effective ART strategy. The data is not enough to compare the overall scenario of the Nepal thus warrant urgency for large scale study with standard genotypic tools.

Keywords: HIV-1, Antiretroviral Therapy, Resistance Mutation, *env* V3 DNA, *RT* gene.

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Introduction

HIV/AIDS is the gradually increasing global epidemic threat with an estimated 36.7 million HIV positive people worldwide. Among them 5.1 million people are in Asia including 3,00,000 newly infected and 1,80,000 AIDS-related deaths occurred in Asia per year [1]. Till date 39,249 people are estimated to be living with HIV in Nepal [2].

The HIV epidemic has changed due to use of antiretroviral therapies (ARTs) that suppress the HIV-1 RNA load in plasma below the detection limit (<50 copies/ml). However, lifelong ART is necessary for patients with latent HIV infections due to decrease in level of CD4+ lymphocytes [3, 4]. The decrease in level of CD4+ T cells caused by HIV infection leads into AIDS and cause opportunistic infections, like Pneumocystis Carinii pneumonia and Kaposi's sarcoma [5]. More than 50% of the worldwide epidemic are due to HIV-1 subtype C and are prone to genotypic resistance with treatment failure. HIV-1 has a higher rate of replication with lack of 3′ to 5′ proof reading mechanism and highly error-prone to reverse transcriptase [6-8]. The selection of drug specific resistance mutation in viral genome is due to treatment failure and those mutations may be archived into the cellular reservoir (Proviral DNA). Thus, mutations can be detected in proviral DNA even if HIV RNA is below detection limit [9]. HIV-1 resistance to ART is a major problem which is due to suboptimum treatment and transmission of resistant variant at the time of infection [10-12].

Resistance mutation is associated with high replicative activities of the viral genome during therapy. Several drugs have been approved for the treatment of HIV-1 infection such as nucleotide and nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), nonnucleoside RT inhibitors (NNRTIs), and fusion inhibitors. However, drug resistance mutations in HIV-1 remain crucial, even though effective (ART) is available in the global market. One of the commonly associated drug resistance mutations in HIV-1 *RT* gene is M184V. The appearance of Lamivudine

(3TC) resistance mutation, M184I, was found to be transiently proceeded to the outgrowth of M184V substitution [13-15]. The study of the nucleotide sequences of both 3TC-resistant variants shows that M184V (GTG) originates from wild-type Met (ATG) but not from the M184I variant (ATA) [16]. Both variants are generated from the wildtype ATG sequence by transitional substitutions (G to A for 184I and A to G for 184V). The G to A substitution is the type of mutation that most commonly occur during replication of HIV-1 thus M184I appears before M184V [17, 18]. In addition, there have been no systematic studies of HIV-1 resistance mutation in Nepalese population.

Here, we performed the preliminary study of common drug resistance mutation, M184V using a basic molecular tool called PCR-RFDA and assessed the detection of proviral DNA in HIV-1 infected patients with prolonged ART.

Materials and Methods

Patients counselling and collection of samples

Individual patient counselling was performed on the background of HIV/AIDs, diagnosis, monitoring, treatment, drug resistant and genotype testing. A questionnaire was performed with regards to drug use, sexual behavior and travel history. 15 samples were collected from HIV-1 infected patients undergoing long term ART commonly with 2-3 NRTIs and 1-2 PIs or an NNRTI for at least 3 - 4 years recruited in sparsha Nepal, sanepa, Kathmandu was included. Out of 15 cases, only two were clinically approved as ART resistance and viral loads of each patients are shown in **Table 1**. Data were collected as a part of a continuing study of antiretroviral treatment at the time of primary HIV-1 infection, which was approved by the Department of Laboratory Medicine, Research Committee of Nobel College and Chief of Sparsha Nepal.

Extraction of Nucleic Acid

The nucleic acid extraction procedure was performed in Biosafety cabinet (BSL-2). HIV-1 RNA was extracted using precision QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) while DNA was extracted using Shine Gene Whole Blood DNA extraction kit (Wuhe Road, Minhang District, Shanghai, China) as per manufacturer's instructions.

Proviral DNA Detection: Nested PCR

Nested PCR for HIV-1 env gene

The nested PCR was performed in the *env* V3 DNA sequences of HIV-1 for the amplification using high-fidelity Pfu DNA Polymerase. The amplification was performed as described in previous study [19]. The total reaction volume of 20µl containing 1X PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, and 0.25 U of Pfu enzyme and RNase free water. The first round PCR was performed with 0.5 µM of upstream primers JA9AE (5′-CACAGTACAATGCACACATG-3′), JA9B (5′-CACAGTACAATGTACACATG-3′), and downstream primers JA12A (5′-GCAATAGAAAAATTCTCCTC-3′), JA12B (5′-ACAGTAGAAAAATTCCCCTC-3′). The reaction mixture was incubated at 95°C for 15 minutes, 94°C for 30 Sec, 58°C for 30 Sec, 72°C for 30 Sec and 72°C for 10 minutes, for 35 PCR cycles. 1 µl of the first-round PCR product was used as template for second-round PCR. Inner upstream primers used were a mixture of 0.33 µM each of JA10UB (5′-CTGTTAAATGGCAGTCTAGC-3′), JA10UC (5′-CTGTTAAATGGTAGTCTAGC-3′), and JA10UG (5′-CTGTTAAATGGCAGTTTAGC-3′). Inner downstream primers used were different for each reaction, namely, 0.33 µM each of JA11LAE (5′-AATTTCTAGATCCCCTCCTG-3′), JA11LB (5′-AATTTCTGGGTCCCCTCCTG-3′), and JA11LC (5′-AATTTCTAGGTCCCCTCCTG-3′). The reaction mixture was incubated at 95°C for 15 minutes, 94°C for 30 Sec, 50°C for 30 Sec, 72°C for 30 Sec and 72°C for 10 minutes, for 35 PCR cycles. The final PCR product was resolved in 1.7% agarose gel electrophoresis and image was captured using gel documentation.

HIV-1 RNA Mutational (M184V) Analysis: PCR-RFDA

RT gene amplification: Nested PCR

The amplification of HIV-1 *RT* gene was performed using nested primer pairs which were reported in previous study [20]. The isolated HIV-1 RNA was subjected to OneStep RT-PCR (Qiagen, Hilden, Germany). The RT-PCR product was then used as templates for second round PCR. The QIAGEN

Table 1: Patient's information, ART and genotypic testing in the study population n-15

Code	Gender/Age	ART used	Viral Load copies/ml	*env* V3 DNA	M184V Mutation	Patient Status
HRM1	Male/36 yrs	AZT, 3TC, NFV	1500	Positive	Negative	Critical
HRM2	Male/33 yrs	AZT, 3TC, EFZ	1500	Positive	Negative	Critical
HRM3	Male/26yrs	AZT, 3TC, NFV	1500	Positive	Negative	Critical
HRM4	Male/35yrs	AZT, 3TC, NFV	1000	Positive	Negative	Expired
HRM5	Male/23yrs	AZT, 3TC, NFV	1000	Positive	Negative	ART
HRM6	Male/32yrs	AZT, 3TC, NFV	300	Negative	Negative	ART
HRM7	Male/34yrs	AZT, 3TC, NFV	800	Positive	Negative	ART
HRM8	Male/25yrs	AZT, 3TC, NFV	800	Positive	Negative	ART
HRM9	Male/28yrs	AZT, 3TC, EFZ	500	Positive	Negative	ART
HRM10	Male/30yrs	AZT, 3TC, NFV	250	Negative	Negative	ART
HRM11	Male/36yrs	AZT, 3TC, NFV	300	Negative	Negative	ART
HRM12	Male/25yrs	AZT, 3TC, NFV	750	Positive	Negative	ART
HRM13	Male/27yrs	AZT, 3TC, NFV	400	Positive	Negative	ART
HRM14	Female/28yrs	AZT, 3TC, EFZ	650	Positive	Negative	ART
HRM15	Female/33yrs	AZT, 3TC, NFV	550	Positive	Negative	ART

Key: HRM-HIV-1 Resistance Mutation, ART-Anti Retroviral Therapy, AZT- Zidovudine, 3TC- Lamivudine, EFV-Efavirenz, NF Nelfinavir

Table 2: Most common mutations in RT gene and specific drugs resistance.

Resistance genes		Resistance level		
Mutation	Gene	High	Intermediate	Low
M184V/I	RT	3TC, FTC	-	ABC, ddI
K65R	RT	ABC, ddI, TNF, d4T	3TC, FTC	
M41L	RT	AZT, d4T	ddI, ABC	TNF
K103N	RT	EFV, NVP	-	-

Key: 3TC- Lamivudine, FTC-emtricitabine, AZT- Zidovudine, d4T- Stavudine, EFV-Efavirenz, NVP- Nevirapine, ABC- Abacavi Didanosine, TNF- Tenofovir

OneStep RT-PCR was performed as recommended by the manufacturer. The total volume of 50µl reaction was carried out containing RNase free water, 1x OneStep RT-PCR buffer, 200 µM dNTP mix, 0.6 µM of each primers A1 (5'-AATTTTCCCATTAGCCCTATT-3') and NE1 (5'-TATGTCATTGACAGTCCAGCT-3'), 2µl of OneStep RT-PCR enzyme mix, 5 units RNase inhibitor and 2 µg viral RNA for reverse-transcriptase gene. The reaction was incubated at 48°C for 20 minutes (reverse transcription) followed by 94°C for 4 minutes, 94°C for 30 Sec, 52°C for 30 Sec, 72°C for 30 Sec, and 72°C for 10 minutes, for 35 PCR cycles. The second round PCR was done by using a QIAGEN master mix with 5 µl of the first RT-PCR product and primers NNA (5'-AAGCCAGGAATGGATGGCCCA-3,) and E (5'-CCATTTATCAGGATGGAGTTC-3'). The reaction mixture was incubated at 95°C for 15 minutes, 94°C for 30 Sec, 50°C for 30 Sec, 72°C for 45 Sec and 72°C for 10 minutes, for 30 PCR cycles.

M184V Mutation Testing: Restriction Fragment Digestion Assay (RFDA)

The amplified *RT* gene was tested for M184V mutation by digestion approach called RFDA. The RFDA was performed using restriction enzymes CviAII. The total volume of 50µl reaction was performed containing 1X NEBuffer, 10 units' CviAII restriction enzyme, and 1µg RT-PCR product at 25°C for 1 hour. The digested product was resolved in 1.7% agarose gel electrophoresis and image were captured using gel documentation.

Results

In the present study, detection of proviral DNA by amplification of *env V3 DNA sequences* was done by nested PCR while testing of resistance mutation in the HIV-1 *RT* (MET 184 VAL) gene was performed by PCR and RFDA. Individual band, resulting from final PCR product and enzyme digested product was compared with ART resistance strain and molecular size marker (100bp). The results are tabulated and is shown in **Table 1**.

Identification of env V3 DNA

The *env* V3 DNA sequence was analyzed to rule out latent HIV-1 infections. Out of 15 samples amplified for *env* gene, only 12 of samples show positive for *env* V3 DNA sequence by nested PCR. The size of the product was 335bp which was a band of our interest and is illustrated in **Figure 1** (a & b). The remaining 3 samples negative for the same gene was reamplified twice showed the similar results. The reason for *env* gene negative in those three samples might be due to complete elimination of proviral DNA or due to its reduction below the detection limit after prolonged ART.

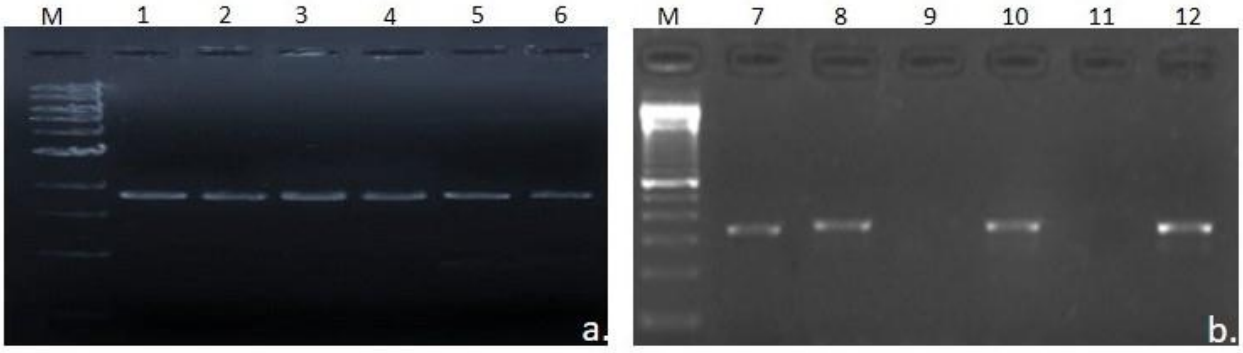

Figure 1: Nested PCR for env V3 DNA sequences. Marker (M) of 100bp was used to compare the molecular weight of amplified product resolved in gel of 1.7%. a) Amplified product with band of interest (335bp) was visualized in all lane 1-6. b) Amplified product was observed only in lane 7, 8, 10, 12 but not in lane 9 and 11. Gel image of 3 positive samples is not shown.

M184V mutation in HIV-1 RT gene

HIV-1 RNA was tested for the most common mutation; M184V which emerges in patients using lamivudine (3TC) monotherapy or can be a phenotypic reversion of zidovudine (ZDV). PCR-RFDA molecular tool was used for testing the mutation in which gene was amplified by nested PCR and further final product was digested with CviAII restriction endonucleases. In the present study, we tested M184V mutation in 15 samples and all showed wildtype strain. The wildtype strains show three fragment after digestion with band size of 453bp, 60bp and 168bp are susceptible to 3TC while mutant type have 2 bands with size of 453bp and 228bp are considered as 3TC resistance which means the M184V resistance mutation was emerged by modification of the 184 restriction site substituting CviAII enzymes. The virtual schematic illustration of Nested PCR and RFDA of both wild type and mutant type is shown in **Figure 2 (a & b)**.

Discussion

Early detection of drug resistance mutation in HIV-1 infected patients in developing countries is a big challenge however, can aid clinicians in making proper ART strategy plans. Various molecular tools like real time PCR and sequencing facilities are still lacking. Also, such tools are expensive and less sensitive to minor resistance variants. The development of cheap and sensitive tools to screen resistance mutation can be a breakthrough in the field of molecular biology for low economic countries like Nepal. In the study, we used nested PCR and PCR-RFDA to detect HIV-1 Infections and screen the prevalence of M184V mutation in HIV-1 patients underwent prolong ART. The cheap and sensitive methods for detecting primary infection in infants and latent HIV-1 infection is to identify proviral DNA. The HIV proviral DNA is positive after 28 days while most healthy individuals are accustomed to waiting 3 months for a conclusive result. We performed nested PCR to identify the *env* V3 DNA sequences in 15 samples, out of which 12 showed positive for proviral DNA. The PCR amplification on 3 negative samples was repeated twice with the same primers for the env V3 DNA but consistently the results were negative. The reason might be a low copy number which is below the detection limit or can be complete elimination of proviral DNA due to prolonged ART. The standard methods for the detection of HIV-1 Proviral DNA are currently lacking and several discordant results are still present in different studies [21]. In the present study, we didn't study for the

Figure 2: Nested PCR and RFDA for detection of M184V resistance mutation. a) Wildtype stain with three bands of size 453bp, 60bp, 168bp. b) Mutant strain with two bands of size 453bp and 228bp

resistance mutation in HIV-1 proviral DNA. However, the presence of drug resistance mutations in the reservoir of latent HIV-1 provirus and their stability may impact future management and/or treatment possibilities. The presence of latent proviral DNA doesn't indicate the resistance to ART but the specific mutation like M184V, k65R, M41L and K103N etc. can be detected if the similar mutation is observed in HIV-1 RNA. This is due to retention of mutant from HIV-1 RNA into cDNA due to long term suppressive ART that integrates into host genome. The evolution of drug resistance mutation in globally epidemic HIV-1 is due to suboptimal

treatment, a point mutation in viral genome and resistance variants leads to incomplete suppression of the viral genome. The development of drug resistance mutation threatens the success of the future therapy regimens. The challenge in treatment of HIV-1 is associated with the development of drug resistance mutation against antiretroviral drugs which can significantly reduce the viral RNA to undetectable levels in plasma [22]. The various resistance mutation pattern has been reported with majorities in both nucleoside reverse transcriptase inhibitors (NRTIs) and non- nucleoside reverse transcriptase inhibitors (NNRTIs) while only a few with NNRTIs and also in minor ratio with protease inhibitors (PI) and 3- class resistance (NRTIs, NNRTIs and PIs). The genotyping of the reverse transcriptase gene sequence revealed that almost 90% resistance mutation were M184V [23] and such mutation emerges in patients receiving lamivudine (3TC) therapy, but also it could be associated with phenotypic reversion of zidovudine (ZDV) resistance [24]. In the present study, we screened for M184V resistance mutation in HIV-1 infected patients receiving 3TC in combination by PCR-RFDA methods. The only wild type strain was found in all the sample analyzed which means the M184V mutation was negative. Our results completely contradict the other reports on M184V. In the study though phenotypic resistance and high viral load were included, M184V mutation was not detected which might be due to numerous reasons like 1) duration of 3TC ART, 2) Possibilities of other mutation like M184I, K65R, etc 3) change in drugs 4) less likely to be technical error . The commonly used drugs by the HIV-1 infected patients in Nepal are AZT, 3TC, EFV and NFV and is shown in Table 1. The high level resistance to 3TC associated with M184V mutation is also resistance to other drugs like FTC, ABC and ddI. In addition to this, resistance to 3TC is also associated with K65R mutation and its variant forms but the level of resistance may vary from high-intermediate-low. The most common RT gene mutation and specific drug resistance is shown in Table 2. There are several mutations in genes of HIV-1 associated with resistance to specific drugs [25]. The M184V mutation was detected in lesser than 10% by allele specific PCR but was consistently negative by standard genotyping [26] While the ratio of mutant and wild type is equal to 50:50 reported by almost half of the laboratories [27]. The prevalence of the M184V mutation is notably higher than that of other mutation [28-30] and is significantly associated with 3TC [31].

The early detection of resistance mutation in HIV-1 infected patients would help to change the treatment regimens susceptible to both Proviral DNA and viral RNA and control measures can be taken to minimize the transmission of resistant variants. An understanding of the molecular mechanism of drug resistance will enable us to develop improved tools for resistance screening.

Conclusion

In the present study, we set out to identify the HIV-1 latent infections by detecting *env* V3 DNA sequences using nested PCR. In addition to this, the M184V resistance mutation was not observed in the samples analyzed thus indicating as wild type strain. Also, we optimized the PCR-RFDA protocol using CviAII restriction enzymes for the HIV-1 RT strains for mutation testing. This strategy was meant to enhance assay discrimination between the specific drug resistance mutation (mutant allele) and its wild type (wild type allele) using nested primers and CviAII restriction enzymes.

Declaration of interests

The authors declare that they have no competing interests.

Authors' contributions

Rupendra came up with the study, frame experimental work and prepared manuscript. All authors have equally involved in completing the research experiment and data compiling. All authors read and approved the final manuscript for publication.

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