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ORIGINAL ARTICLE

Subtype diversity and emergence of drug resistance in HIV-1 in solapur district of Maharashtra, India

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ABSTRACT

Background and Objectives: Even after four decades, HIV infection remains a global challenge and a leading cause of mortality in adults across the world. Anti-retroviral therapy (ART) that controls HIV viremia, is now available through public health facilities in India but drug resistance, which is likely to develop among these individuals remains poorly studied in India. The objectives of present study are to find out the HIV-1 virus subtypes, drug resistance mutations and HIV-1 drug resistance to NRTI, NNRTI and protease inhibitors in the Solapur district, India.

Materials and Methods: In a cross sectional study, forty two ART-experienced HIV-1-infected patients with CD4+ count < 200 cells ml-1 and viral load (VL) > 3, 000 copies ml-1 were recruited. All patients belonged to Maharashtra State of India near Barshi Solapur and had been on ART treatment for over 5 years. EDTA whole blood from HIV-1-infected patients was centrifuged and the viral nucleic acid was purified from the plasma. Viral nucleic acid was amplified by PCR using protease and reverse transcriptase specific primers. The resulting amplicons were sequenced and studied for mutations. The tools from Stanford University website were used for subtyping of HIV-1 and identification of mutations conferring drug resistance.

Results: In present investigation, HIV-1 subtypes were subtype C in 37 (88.09%), subtype CRF01_AE in 2 (4.76%), and subtype A in 3 patients (7.14%). Drug resistance mutations of NRTI, NNRTI and protease were observed in 15 (37.71%) of 42 patients tested. Drug resistance for NRTI was observed in 12 (28.57%) and for NNRTI in 13 (30.95%) patients. No drug resistance was observed for protease inhibitors.

Conclusion: Considerable HIV-1 drug resistance exists among patients receiving ART from a rural areas of India, suggesting more studies from rural region are required to prevent development of resistance to ART.

Keywords: HIV-1; Antiretroviral therapy; CD4+ count; Viral load; Drug resistance

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INTRODUCTION

Current advancements in Anti Retroviral Therapy (ART) have bowed HIV-1 infection into a chronic and manageable disease (1). HIV treatment programmes have saved millions of lives, prevented millions of infections and restored hope to the populations that have been hardest hit by the pandemic. Curiously, treatment is only effective until the HIV-1 develops resistance against the ART drugs. However, the challenges of treating HIV-1 are constantly evolving (1) and HIV/AIDS remains a significant global problem. There is increasing evidence that HIV/AIDS pandemic is declining among general populations worldwide. The epidemiology of HIV infection is changing, dynamic, complex and progress in epidemic control remain markedly uneven. Current efforts are unlikely to succeed without addressing the components of global HIV/AIDS spread (2).

In 2014, the Joint United Nations Programme on HIV/AIDS (UNAIDS) (3) launched a global project on HIV/AIDS to end the HIV epidemic by 2030. At present it is building a new narrative on HIV treatment and a final, ambitious, but achievable targets , a) 90% HIV infected patients will know their HIV status b) 90% HIV patients will receive sustained antiretroviral therapy (ART) and 90% patients receiving ART will experience substantial viral suppression. However, wide use of ART is also likely to promote the emergence and spread of HIV drug resistance, this becomes even more pronouncing when therapy is empirical and/or inaccurately prescribed. Recommending an appropriate HIV Drug-Resistance Testing (DRT) method is crucial in the surveillance and prevention of HIV in the China (4). The HIV-1 drug resistant mutations in India are described in detail by Karade et al., 2018 (5). Periodic monitoring is essential in drug resistance studies against first-line ART agents. It is also recommended that there is a need to develop an HIV-1 subtype C-specific resistance database in India (6). There is a paucity of information on the account of subtype diversity and drug resistance types and frequency among the patient receiving first line of ART treatment from the rural part of India. An efficient and aggressive reporting on the serotyping of subtype diversity and drug efficacy through estimating drug resistace frequency is a need of hour for achieving the UNAIDS goal to end HIV by 2030.

We sought to find out HIV-1 drug resistance in the rural and remote area of this investigation, Patients with history of ART treatment more than 5 years (Inclusion & Exclusion criterion), are being considered to estimate HIV-1 subtype diversity and sensitivity/ resistance against first line drugs treated at the rural ART centers. Objectives of this investigation were to find out; 1. HIV-1 drug resistance frequency among adults receiving first line therapy at the ART 2. to estimate HIV subtype diversity in this study area, 3. decipher mutations in the reverse transcriptase (RT), protease gene a part of *Pol* region of the HIV-1, and 4. drug resistance to Nucleoside reverse transcriptase inhibitors (NRTI), Non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors.

MATERIALS AND METHODS

Selection of study population. In this observational cross sectional study, forty-two HIV-infected patients (25 females and 17 males) on ART were counselled to participate in this study. All the patients were married and heterosexuals belonging to middle and/ or low income groups from agriculture field. All the individuals were on ART treatment for >5 years, have low CD4 count and VL > 3000 copies ml-1 and were on the terminal stage of illness while being treated with first line ART therapy. These individuals were selected from 913 patients under HIV treatment. Due to lack of funds, individuals only on ART treatment for more than 5 years were selected. Patients qualifying above mentioned criteria 42 were considered for nucleotide sequencing. Besides treatment failure, VL and drug resistance studies were not performed routinely in this area. Treatment was monitored only based on CD4 count. There was no surveillance for drug resistance due to lack of VL facility. The study was very important to inform policy concerning the status of resistance to ART treament in Solapur district, it was of paramount importance because drug resistance testing has not routinely done for patients receiving first-line therapy at the ART. The patient's full information, viz-a-viz; demographic, clinical and laboratory information was recorded on standardized sheets. All the patients had signed an informed consent form towards their participation in this study. The study was conducted at Barshi Solapur, Maharashtra State, India, from April 2016 to May 2018. The genotypic study of the patients from 1 to 25 was done on 11/05/2016 and the samples from 101 to 117 on 25/05/2016.

Blood samples. Four ml of blood was collected in an EDTA vacutainer from the median cubital vein of the elbow crease. The blood was then centrifuged at 1000 g for 10 min and clear plasma was obtained. Two ml of plasma frozen in dry ice was sent to the SRL Diagnostic Limited, Mumbai, for analysis of VL and sequencing of PR and RT genes. CD4+ count was analyzed with the FACS Count (Becton Dickinson Immunocytometry Systems) at the SRL Diagnostic Ltd, Mumbai. Whole blood EDTA and Heparin were sent at ambient temperature for determination of CD4+ count. Selection of patients was done based on CD4 count, less than 200 cells ml^{-1}. EDTA plasma was collected for PCR and genotypic study of the selected patients.

Extraction of RNA, amplification and sequencing. In this study protocols used for the HIV-1 drug resistance studies are described in detail in Manasa et al. (7). RNA were extracted from the patient blood using QIAamp RNA blood mini kit procured from Qiagen (Germany). Upon confirming RNA quality one-step RT-PCR was carried out using Qiagen one step RT-PCR kit, Qiagen (Germany) and primers, which were selected from previously described by Steegen et al. (8). The RT-PCR products; 300bp for Protease and 800 bp for Reverse Transcriptase were processed for PCR product cleanup.

Amplicons were used to decipher nucleotide sequence from both the strands. Sequences from the study had been submitted to GeneBank and are available with accession numbers MK318919 and MK331889-MK331929.

Construction of phylogenetic tree. A phylogenetic tree was constructed using MEGAX. 43 HIV sequences and 3 reference sequences were aligned by MSA (Multiple Sequence Alignment) tool ClustalW in MEGAX. A rooted phylogenetic tree was generated by taking subtype C as root using maximum likelihood method based on general time reversible model with bootstrap analysis of 1000 repetitions, a rooted phylogenetic tree was created and is depicted in the figure. The subtype C reference sequence is being represented by the blue box, while reference sequence for Subtype A and CRF is denoted by the thick red upward arrow. In order to produce the rooted phylogenetic tree, we used Subtype C as the root node. All sequences depicting diversity yet belonging to the subtype C, shown in green and yellow box.

HIV-1 RNA PCR. HIV RNA was detected using Polymerase chain reaction (PCR) method as described by Holmes et al. (9). This was done to confirm the HIV status of the patient. An aliquot of 2 ml EDTA plasma was collected from the selected patients and sent frozen in dry ice to SRL Diagnostic Ltd, Mumbai.

Viral genetic subtyping. Subtyping of HIV-1 sequences and drug resistance analysis was done as described by Liu et al. 2006 (10). Representative subtype reference sequences of HIV-1 group M subtypes A, B, C, and CRF01_AE were used from Leitner (11). MEGA version 10.0.5 tool was used for the phylogenetic analysis and phylogenetic tree drawing (12). The evolutionary history was inferred by using the Maximum Likelihood method and General time reversible model. The percentage of trees with associated taxa clustered are being shown next to the branches. Initial tree(s) for the exploratory search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix while pairwise distances were estimated using the Maximum Composite Likelihood (MCL) approach.

Determination of drug resistance. Drug resistance mutations were examined by using the Stanford University HIV drug resistance database website https://hivdb.stanford.edu. The programme analyses sequences for resistance mutations listed in the WHO website updated periodically (13). The list of HIV-1 drug resistance mutation of NRTI, NNRTI, and PI which was used as reference mutations included in the Stanford University drug resistance mutation database. These mutations are known to cause HIV-1 drug resistance in the patients, which results in ART failure (14).

Ethical considerations. Ethical committee was formed as per guidelines of the Indian Council of Medical Research (ICMR). The study was approved by the ethical committee of Shri Shivaji Mahavidyalaya, Barshi. The signed informed consent was gained from each participanting subjects (15).

RESULTS

Forty-two patients with median CD4+ count 145 and median VL 84,172 copies ml^{-1} were selected to

find out drug resistance mutation studies and HIV-1 subtypes. Out of 42 HIV patients, 25 were females and 17 were males with median age 31 years. The important part of the study is that the origin of the samples is a rural part of India and gives deeper picture of HIV strains of India. The sequences used as input to the Stanford University database and mutations conferring resistance were deciphered. The HIV-1 subtypes were also detected by using tools available on the website of Stanford University database https://hivdb.stanford.edu/. Table 1 shows Sequence ID, Gene Bank Accession Number, sequence length in bp, sex of the patient, CD4+ count, VL, first line ART duration in years, HIV-1 subtype, and No. of drug resistance mutations found in the sequenced isolate of the HIV-1 patient under treatment. In present investigation, following HIV-1 subtypes were found; subtype C in 37 (88.09%) sequence isolates of HIV-1 patients, subtype CRF01_AE in 2 (4.76%) and subtype A in 3 patients (7.14%) shown in Fig. 1. Table 1 shows NRTI, NNRTI, and protease mutations were detected in 15 out of 42 patients tested. A protease inhibitor mutation was observed in one patient (Q58E). Mutation Q58E has not been known to impart drug resistance against the drug targeting PI, hence, drug resistance against protease inhibitor was considered to be Nil.

Subtype diversity -phylogenetic tree. We have demonstrated groups in the phylogenetic tree, Fig. 1, in which various clusters are shown properly with bootstrap values for Subtype C, Subtype A and CRF01_AE. The tree is representing 3 cluster with their refernce sequence. As represented in the tree, the sequence 25,113,15,A,21, CRF01_AE, 4 are distant from the root, so we can say these sequences are sequentially different from the Subtype C (root). Further, these sequences are clustered in two groups each representing subtypes A and subtypes CRF01_AE with high cofidence as represented by bootstrap value of the corresponding node. Rest of the sequences are much more close to the Subtype C. Furthermore, according to the bootstrap value of the relevant node, these sequences are strongly grouped into two groups, each of which represents the subtypes A and CRF01_AE, shown in Red box Fig. 1. The remaining sequences are substantially closer to subtype C, shown in green and yellow box Fig. 1.

HIV-1 drug resistance. Total 23 types of mutations were observed of which 12 mutations were of NRTI, 10 of NNRTI and 1 of protease inhibitor gene. Total 20 mutations of NRTI type were found in 42 patients and 36 NNRTI mutations in 14 patients (Table 1 and Fig. 2). Twenty-five patients did not have any NRTI, NNRTI, and protease inhibitor mutations. Hence they were susceptible to all the ART drugs (Fig 2). Table 2 shows NRTI drug resistance of the patients (n=42) due to presence of drug resistance mutations. In the present investigation, overall 35.71% drug resistance was observed out of which 28.57% resistance was seen for NRTI drugs.

It shows high level, intermittent drug resistance to NRTI drugs used for the treatment of HIV-1 patients. The incidence of resistance to abacavir (ABC), zidovudine (AZT), emtricitabine (FTC), lamivudine (3TC), and tenofovir (TDF) is shown in Table 3. Presence of a single mutation in the sequenced isolate is treated as resistance even if it has high level, intermediate or low level drug resistance. Maximum NRTI drug resistance (26.19%) was observed for abacavir (ABC), followed by lamivudine (3TC) (23.81), emtricitabine (FTC) 21.43%. Lowest drug resistance observed was for zidovudine (19.05%) and tenofovir (14.29%) (Table 3). Table 3 shows Predicted drug resistance for NNRTI drug resistance mutations scores in patients (n= 42). Number of patients who showed NNRTI mutations were 13 while the remaining 29 patients did not show any NNRTI mutations, hence susceptible to NNRTI drugs.

NRTI drug resistance was observed for abacavir (ABC) in 11 (26.19%), zidovudine (AZT) 8 (19.05%), emtricitabine (FTC) 9 (21.43%), lamivudine (3TC) 10 (23.81), and tenofovir (TDF) 6 (14.29%) patients. For NNRTI drugs resistance was observed for doravirine (DOR) 12 (28.57%), efavirenz (EFV) 13 (30.95%). etravirine (ETR) 11 (26.19%), nevirapine (NVP) 13 (30.95%), and rilpivirine (RPV) 11 (26.19%) patients (Table 3). This prediction of drug resistance was done by mutation scoring by HIVdb programme as per Stanford University HIV mutations database programme and method was described by Rhee et al. 2009 (16). Forty-two out of 913 patients qualified inclusion & exclusion criterion those wee subjected for deciphering nucleotide sequences for reverse transcriptase and protease inhibitor. The five drug resistance levels were observed (Table 3). There are five ranks of resistance:1 susceptible, 2 potential low-level resistance, 3 low-level resistance, 4 intermediate resistance, 5 high-level resistance

Table 1. Subtype and nucleotide sequence

Sequence ID	GeneBank Accession Number	Length bp	Sex	CD4+ count	VL (copies per ml)	Sequence ID	First- Line ART duration in Years	HIV-1 Subtype	No. of drug resistance mutations
1-P058494	MK318919	655	F	159	13600	1-P058494	7	C	0
2-P026490	MK331889	607	M	162	39363	2-P026490	9	C	0
3-P026476	MK331890	685	M	150	61400	3-P026476	6	C	0
4-P046865	MK331891	618	F	152	>750000	4-P046865	8	CRF01_AE	4
5-P046901	MK331892	922	M	34	618000	5-P046901	14	C	0
6-P026427	MK331893	1136	M	166	387828	6-P026427	16	C	3
7-P026495	MK331894	731	F	187	150548	7-P026495	6	C	7
8-P026509	MK331895	687	F	136	111000	8-P026509	9	C	3
9-P026523	MK331896	733	F	55	515000	9-P026523	12	C	0
10-P046910	MK331897	766	M	157	366321	10-P046910	15	C	6
11-P046908	MK331898	825	F	46	120773	11-P046908	13	C	0
12-P026472	MK331899	806	F	21	84172	12-P026472	13	C	0
13-P046886	MK331900	754	F	83	99800	13-P046886	11	C	0
14-P026439	MK331901	711	M	179	103090	14-P026439	16	C	0
15-P046926	MK331902	926	F	187	150500	15-P046926	14	A	0
16-P046432	MK331903	661	M	174	341000	16-P046432	9	C	0
17-P046851	MK331904	902	M	143	460431	17-P046851	10	C	2
18-P046878	MK331905	919	M	140	220000	18-P046878	5	C	4
19-P026499	MK331906	708	M	12	>750000	19-P026499	13	C	0
20-P046878	MK331907	903	F	152	>750000	20-P046878	15	C	0
21-P046896	MK331908	630	F	21	722660	21-P046896	17	CRF01_AE	4
22-P026590	MK331909	934	F	83	99800	22-P026590	6	C	1
23_P026591	MK331910	1302	M	186	11400	23_P026591	11	C	0
24_P026592	MK331911	1286	M	127	15600	24_P026592	8	C	0
25_P026593	MK331912	1065	M	116	45000	25_P026593	7	A	0
101_26112018	MK331913	962	F	90	4000	101_26112018	6	C	0
102_26112018	MK331914	770	F	123	35000	102_26112018	7	C	3
103_26112018	MK331915	993	F	148	3500	103_26112018	5	C	0
104_26112018	MK331916	1053	M	121	12000	104_26112018	7	C	0
105_26112018	MK331917	895	F	183	15210	105_26112018	5	C	0
106_26112018	MK331918	843	F	79	32000	106_26112018	5	C	0
107_26112018	MK331919	1060	F	177	>750000	107_26112018	10	C	7
108_26112018	MK331920	770	M	154	18000	108_26112018	9	C	0
109_26112018	MK331921	1004	F	133	4600	109_26112018	8	C	0
110_26112018	MK331922	1065	F	148	80000	110_26112018	6	C	4
111_26112018	MK331923	1190	F	112	3610	111_26112018	5	C	0
112_26112018	MK331924	906	F	161	4500	112_26112018	7	C	0
113_26112018	MK331925	858	M	108	15000	113_26112018	8	A	0
114_26112018	MK331926	712	F	200	35000	114_26112018	8	C	1
115_26112018	MK331927	693	M	142	55000	115_26112018	5	C	0
116_26112018	MK331928	686	F	182	85000	116_26112018	6	C	3
117_26112018	MK331929	767	F	168	>750000	117_26112018	6	C	6

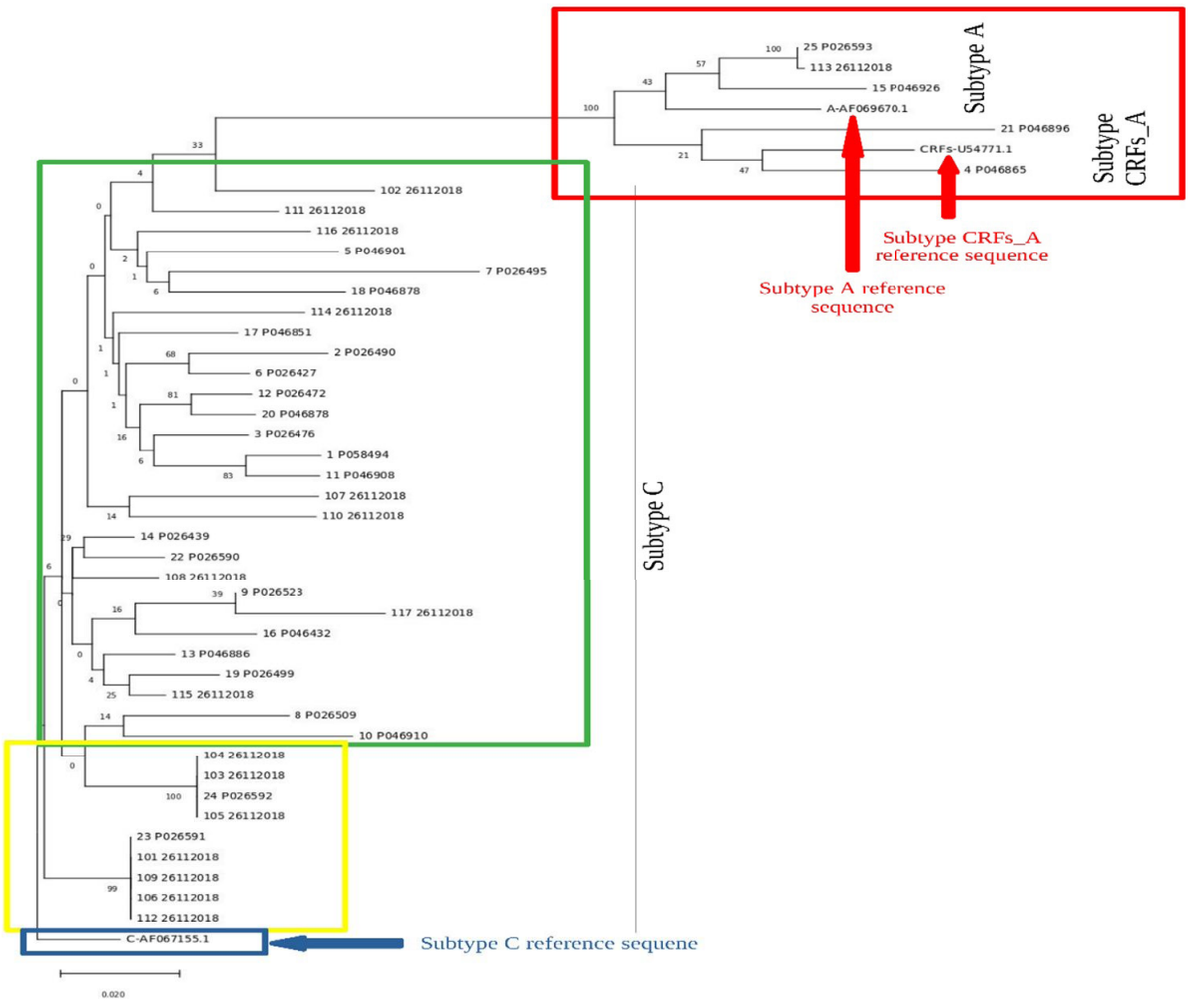

Fig. 1. HIV-1 subtype diversity and Phylogeny using reference sequences. Three distinct clusters were found of subtype A, subtype C and subtype Circulating recombinant forms (CRFs) CRF01_AE in comparison with all the reference sequences of HIV-1 subtypes. Sequences comprising the complete PR and partial RT genes of test viruses were aligned using MEGA version.10.0.5. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 45 nucleotide sequences; 42 sequences obtained from patients while three references from the HIV database. Evolutionary analyses were conducted in MEGA X as published in Kumar et al. in 2018 (11).

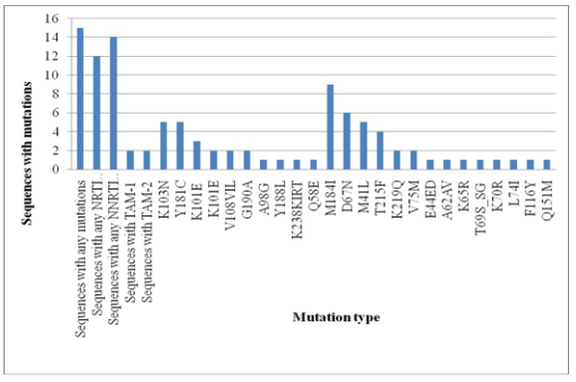

Fig. 2. Sequences with NRTI and NNRTI mutations in patients (n=42). Thymidine analog resistance mutations, TAM-1 (41L, 210W, 215Y) and TAM-2 (67N, 70R, 215F, 219E/Q) are NRTI mutations

(https://www.stanford.edu/). The score represents the total of each mutation penalty score for the drug. Score less than 10 indicate susceptibility; score between 10 to 14 indicate potential low-level resistance; score between 15 and 29 indicate low-level resistance; score between 30 and 59 indicate intermediate resistance, High-level resistance is indicated for score of 60 or higher.

We found low level drug resistance with a penalty score of 0-9 against NRTI drugs; abacavir, ABC (1), zidovudine, AZT (3), emtricitabine, FTC (2), lamivudine, 3TC (0) and tenofovir, TDF (1) and for NNRTI doravirine, DOR (0), efavirenz, EFV (0), etravirine, ETR (2), nevirapine, NVP (0) and rilpivirine, RPV (2).

If two mutations occur at the same time at the same

base then the mutation imparting highest penalty was scored. Interestingly, negative penalty score is nullified if a mutation was associated within a mixture with the wild type amino acid at that position (https://www.stanford.edu/). Negative penalty score (< 0) for NRTI drug; zidovudine, AZT (1), lamivudine, 3TC (2) and tenofovir, TDF (4) and NNRTI negative penalty score was observed only for doravirine, DOR (1).

DISCUSSION

In this study, the prevalence of first-line ART resistance mutations in patients with HIV/AIDS was analyzed in April and May 2016 conducted in Barshi, Dist Solapur of Maharashtra. In this region, HIV treatment is being monitored only by CD4 count. The HIV drug resistance is an ambient report from this area i.e. rural Maharashtra. As, VL and HIV drug resistance testing is not included in the surveillance of HIV patients in this region, success or inefficacy of ART treatment surveillance remains unknown. VL monitoring in this setting is crucial and individual genotypes must be determined in case of failure of first-line therapy. The drug resistance was observed in the individuals undertaken first-line of ART treatment in the area of investigation. The ultimate goal of the UNAIDS Control Programme is to bring significant reduction and reversion of pandemic severity, which is greatly threatened by ever growing drug resistance challenge. In India, the free ART programme increased from eight centres in 2014 to over 519 centres in 2015 (17), but rural part of Maharashtra is yet to find place in the surveillance programme.

We have demonstrated groups in the phylogenetic tree, Fig. 1, in which various clusters are shown properly with bootstrap values for Subtype C, Subtype A and CRF01_AE. Overall, our research demonstrates that the subtypes A and CRF01_AE are highly distinct from one another, unrelated to subtype C, yet they are more closely related to one another in terms of evolutionary distance than the subtype C. The patient sequences represented by the box in yellow are distinct from those of the Subtype C yet have similarities with other patient sequences; as a result, they cluster together and have a high degree of similarity. The patient sequences that are represented by the green box are those that are not only distinguishable

Table 2. NRTI, NNRTI, and protease inhibitor mutations in patients (n=14) showed mutation to either NRTI or NNRTI drugs.

Sequence ID	HIV-1 Subtype	NRTI Mutations	NRTI drug resistance	NNRTI Mutations	NNRTI drug resistance
4_P046865	CRF01_AE	D67N, K70R, M184V, K219Q	ABC, AZT, FTC, 3TC, TDF	None	Nil
6-P026427	C	None	Nil	K103N, V108VIL, K238KIRT	DOR, EFV,NVP
7-P026495	C	M41L, D67S, T69S_SG, V75M, M184V, T215F	ABC, FTC,3TC	Y181I	DOR, EFV, ETR, NVP, RPV
8-P026509	C	D67N, M184V	3TC, ABC, DDI, FTC ABC,	Y181C K103S,	DOR, EFV, ETR, NVP, RPV
10-P046910	C	A62V, T69S_SA, M184V, T215F	AZT, FTC, 3TC, TDF ABC,	G190A Y188L	EFV, ETR, NVP, RPV
17_P046851	C	M184V M41L,	FTC, 3TC	A98G, K103N	DOR, EFV, ETR, NVP, RPV
18-P046878	C	M184V	ABC, FTC, 3TC,	K101E, G190A	DOR, EFV, ETR, NVP, RPV
21-P046896	A	D67N, L74I, T215F, K219Q	ABC, AZT, TDF	K101E	DOR, EFV, ETR, NVP, RPV
22-P026590	C	None	Nil	K101E	DOR, EFV, ETR, NVP, RPV
102_26112018	C	M41L, E44ED	AZT	K103N, V108I	DOR, EFV, ETR, NVP, RPV
107_26112018	C	M41L, D67DN, V75M, M184V, T215Y	ABC, AZT, FTC, 3TC, TDF	Y181C	DOR, EFV, NVP
110_26112018	C	M41L, M184V, T215F	ABC, AZT, FTC, 3TC, TDF	Y181C	DOR, EFV, ETR, NVP, RPV
116_26112018	C	D67N, M184V	ABC, FTC, 3TC	Y181V	DOR, EFV, ETR, NVP, RPV
117_26112018	C	A62AV, K65R, F116Y, Q151M, M184I	ABC, AZT, FTC, 3TC, TDF		DOR, EFV, ETR, NVP, RPV

Table 3. Predicted drug Resistance of Nucleoside reverse transcriptase inhibitor (NRTI) drug resistance mutation scoring in the patients (n=42). No. of patients showed NRTI mutations (12), NNRTI (13).

Sequence ID	NRTI drug resistance mutation scoring						NNRTI drug resistance mutations scoring					
	No. of NRTI Mutations	ABC	AZT	FTC	3TC	TDF	No. of NNRTI Mutations	DOR	EFV	ETR	NVP	RPV
4-P046865	11	60	55	70	70	15	3	10	100	0	105	0
7-P026495	8	110	135	95	95	80	1	20	30	60	60	60
8-P026509	2	20	5	60	60	-5	1	10	30	30	60	45
10-P046910	4	90	95	95	95	65	2	0	90	10	120	15
17-P046851	1	15	-10	60	60	-10	1	60	60	10	60	60
18-P046878	2	20	5	60	60	-10	2	15	75	10	90	15
21-P046896	7	55	70	0	0	30	4	20	60	30	90	60
22- P026590	0	--	--	--	--	--	1	15	15	15	30	45
102-26112018	1	5	15	0	0	5	1	15	15	15	30	45
107-26112018	10	55	85	70	70	25	2	10	70	0	75	0
110-26112018	5	45	55	65	65	15	1	10	30	30	60	45
116-26112018	2	20	5	60	60	-5	1	10	30	30	60	45
117-26112018	9	135	70	115	115	95	1	20	30	60	60	60

NRTI drugs; Abacavir (ABC), Zidovudine (AZT), Emtricitabine (ETC), Lamivudine (3TC), Tenofovir (TDF). NNRTI drugs; Doravirine (DOR), Efavirenz (EFV), Etravirine (ETR), Nevirapine (NVP), Rilpivirine (RPV). Resistance Scoring matrix: Susceptible 0 to 9, Potential low level resistance 10 to 14, Low level resistance 15 to 29, Intermediate resistance 30 to 59, High level resistance >60

from the subtype C, but also do not exhibit much similarities among themselves. As a result, these sequences do not cluster together, and because they do not demonstrate any significant value, hence they appear to be random sequences. A great degree of sequence diversity was seen in the most common serotype, subtype C.

We found K103N, Y181C NRTI and M184I, D67N, M41L and T129Q NNRTI mutations along with other mutations causing drug resistance. In HIV- 1 subtype C-infected patients who failed tenofovir-based regimens, studies have found a significant incidence of K65R (18). The shift in HIV treatment strategy has had an impact on the prevalence of drug-resistant viruses in the population as well as the spread of resistant viral species (19).

In present investigation, HIV-1 subtypes found were subtype C in 37 (88.09%) sequence isolates of HIV-1 patients subtype CRF01_AE in 2 (4.76%) and subtype A in 3 patients (7.14%) as shown in Fig 1. Subtype C is the reason for the Indian HIV epidemic, in Pakistan, Southern Africa, Eastern Africa, India, Nepal, and parts of China, also subtype C is the dominant form. Although differences in subtype do not pinpoint which antiretroviral therapeutics be chosen, however, inappropriate antiretroviral therapy within subtype C is more likely to impart Drug Resistance Mutations (DRM) due to increased selection pressure (20). Protease inhibitor mutation was found in 1 individual. This prevalence is consistent with the other finding in the literature. There was no drug resistance observed in the northern part of India (21). In Kakinada, India it was 2·1% for NNRTI and no drug resistance was observed for NRTI drugs (22). It was found in clinics in Chennai and Mumbai that overall drug resistance was 5·2%, 4.2% for NRTI and NNRTI each (23). In Mumbai, overall drug resistance was 10%, for NRTI 7·5%, and no drug resistance was observed for NNRTI drugs (24). In present investigation, we found overall 35.71% drug resistance to ART drugs. Drug resistance to protease inhibitor was 1% (21) and 2·5% (24). Although, overall drug resistance being reported in this study is relatively higher than earlier reports, it is worth taking a note that this is a first study from the rural part and there has been a gap of 5 to 10 years between the reports and this study. Continuous reporting on seroprevalane, subtype diversity and drug resistanc will help to understand if the drug resistance against ART is on decline, stable or enhanced from the peri-

od of earlier reports.

Resistance development among the ARV-exposed group unlikely be avoided, however, optimal adherence and monitoring can be delayed. During 2004–2014, the only NRTI and NNRTI mutations that showed rising trend for K65R and K103N (25). In the study, it was evaluated that HIV-1 drug resistance was found in pre-treatment and those failing first-line NNRTI-based ART in South Africa. Patients failing first-line ART most often developed resistance to NNRTIs.

In India and as well as developing countries, detection of prevalent DRM would be a cost-effective alternative over the RT sequencing when it comes to HIV drug resistance genotyping for all patients who have failed to respond to the treatment. In Indian people living with HIV (PLWHA), the most common NNRTI variants were K103N, Y181C, and G190A. This study as well identified these mutant alleles conferring ART resistance. As a result, screening for these variants is highly recommended, which might serve as a cost-effective alternative. HIV-1 subtype C is a prevalent subtype in India and South Africa accounts for nearly 50% of infections worldwide (25).

These studies were limited to an area and it may not be indicative of the rest of India because the drug resistance testing is not regularly offered as a part of the programme. This study was not funded by any agency hence imparted limitations leading to deciphering sequencing from 42 patients and screening them for HIV drug resistance studies. Rural India represents a diverse socio-cultural backgrounds, poor access to healthcare facilities for an individual as well as remoteness of the location. This emphasizes a need to investigate resistance patterns in the rural and suburban parts of India. Improvements in sample transfer protocols from peripheral ART centers, as well as dried blood spots usage, may expand the reach for testing resistance more than before (26).

During the past 20 years, the importance of HIV-1 drug resistance has taken on a new meaning. Great steps in antiviral drug development have led to many effective treatments. WHO is dedicated to ensuring that the scale-up of HIV-1 treatment in the last decade is not jeopardized by the emergence and spread of HIVDR. The application of WHO guidelines has increased the global risk of HIVDR even further (27), recommending "Treat All" and prophylaxis before exposure, and many more people initiating HIV-1 treatment (28). While fears of resistance should not prevent ART from being provided to everyone who seeks it, the long-term effects of earlier initiation on adherence and medication resistance must be constantly evaluated and addressed (27).

The purpose of every ART programme is to maintain the effectiveness of first-line ART by monitoring HIVDR and implementing effective responses. The limitation of our study is the small sample size and restricted study areas that may only represent rural scenarios from western Maharashtra, India. Follow up studies involving larger sample size covering various rural parts of India are desired (28).

CONCLUSION

Based on the results and analysis of this study, we recommend that VL and HIV-1 drug resistance testing must be included in the impact assessment of ART in rural and subrural parts in India. At the infection stage, an individual may get a drug resistant virus infection. Hence we recommend drug resistance studies must be done at the infection stage or at any time when the HIV-1 status is identified. Drug naive individuals have lower risk of virological failures but drug resistant HIV-1 infection is likely to result treatment failure. Hence drug resistance studies must be done after the diagnosis of HIV-1 eventhough patient is asymptomatic.

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