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MOLECULAR EPIDEMIOLOGY AND DRUG RESISTANCE SURVEY OF HIV-1 AMONG ANTIRETROVIRAL NAIVE PATIENTS IN MUMBAI, INDIA.

By

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
AZT	Zidovudine
CRF	Circulating Recombinant Form
d4T	Stavudine
DBS	Dry Blood Spot
DNA	Deoxyribonucleic Acid
DPS	Dry Plasma Spot
DRM	Drug Resistance Mutation
EFV	Efavirenz
FSW	Female Sex Worker
HAART	Highly Active Antiretroviral Therapy
HIV 1	Human Immunodeficiency Virust type 1
HIV 2	Human Immunodeficiency Virust type 1
HIVDR	HIV Drug Resistance Survey
IDU	Injecting Drug User
MSM	Men who have Sex with Men
NACO	National AIDS Control Organization
NNRTIs	Non- Nucleos(t)ide Reverse Transcriptase Inhibitors
NRTIs	Nucleos(t)ide Reverse Transcriptase Inhibitors
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PIs	Protease Inhibitors
PLHAs	People Living with HIV AIDS
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SDRM	Surveillance Drug Resistance Mutation

STD	Sexually Transmitted Disease
3TC	Lamivudine
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization

LIST OF ABBREVIATIONS OF AMINO ACIDS

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

EXECUTIVE SUMMARY

The study on molecular epidemiology of HIV 1 contributes for better understanding of the evolution and genetic diversity of the virus. A regular population based survey of HIV 1 drug resistance among antiretroviral naïve patients would be useful to assess failure in HIV prevention and treatment programs, and to make adjustment in ARV programs for maximum efficiency of limited HAART regimens available in resource limited settings of the world. Present study aimed to understand the genetic diversity and measure the prevalence of drug resistance to NRTIs, NNRTIs and PIs, among antiretroviral naïve patients recruited in Mumbai, India in 2009-2010. The study involved the amplification and nucleotide sequencing of HIV 1 for reverse transcriptase and protease gene, from the DPS samples collected on Whatman 903 paper in Mumbai. The drug resistance mutations were interpreted according to WHO SDRMs list, and subtyping was performed by Phylogenetic analysis using software Clustal W 1.83.

Among a total of 68 samples processed, 52 samples could be amplified on reverse transcriptase and protease gene. Of these 52 amplified products, 51 could be sequenced for reverse transcriptase gene and all of the 52 were sequenced for protease gene. Phylogenetic analysis of sequences revealed all the isolates belonging to subtype C virus, except one which was found to be A-C intersubtype recombinant form. Resistance to antiretroviral drugs was observed to NNRTIs in position K103N, and V106M and to PIs in position M46I. Two of the isolates had V106M mutation, one isolate had K103N and V106M mutation, one had K103N as well as M46I mutation and one had only M46I mutation. So, the prevalence of resistance to drug class NNRTI was 7.8% and to PIs was 3.8%. The overall prevalence of drug resistance to NRTIs, NNRTIs, and PIs is about 10% which is within the WHO threshold of 5-15%. Substantial polymorphism was observed compared to subtype B reference virus, however the polymorphism pattern in HIV 1 isolates from Mumbai seems to be stable when compared to previous studies.

The HIV 1 epidemic in Mumbai is predominantly caused by subtype C, although there is evidence of low level circulation of A-C intersubtype recombinant forms. The 10% overall prevalence of drug resistance to NRTIs, NNRTIs and PIs urges the ARV program authorities in Mumbai to take timely preventive actions for minimizing the emergence and transmission of drug resistant viruses and to study for possible adjustment in programs to prevent treatment failure in first line HAART.

Key words: HIV 1, molecular epidemiology, drug resistance, Mumbai.

CHAPTER I

1. INTRODUCTION

The availability and accessibility to Highly Active Antiretroviral therapy (HAART) and its efficient use has greatly reduced the morbidity and mortality related to HIV -1 infection (Jaggy *et al*., 2003, Palella *et al*., 1998). However, with the use of currently available drugs, the emergence of HIV drug resistance is inevitable in patient under therapy due to the selection of drug resistant mutants, ultimately limiting the success of treatment (Tamalet *et al*., 2003, Richman *et al*., 2004). Failures in treatment and prevention programs can lead to the transmission of drug resistant viruses in the people who are newly acquiring the HIV infection (Masquelier *et al*., 1993, Hermans *et al*., 1993). Regular population based survey of transmission of drug resistant strains is important to assure continued efficiency of the limited ARV regimens accessible in resource limited countries (Bennett *et al*., 2008). Circulation of drug resistant strains of HIV -1 in newly infected people raises serious concerns, as it indicates the failure in treatment and prevention programs, limits the use of fixed antiretroviral regimens in use and seriously affects the ARV programs in resource poor settings where the routine availability of drug resistance testing does not exist. Ultimately, it might drive the scenario in which there would be serious problems in availability of effective drugs for HIV treatment.

Increasing amount of data in molecular epidemiology and drug resistance of HIV -1 is being available from researches in many countries worldwide, because of its implications in treatment, prevention, understanding of epidemiological linkages and vaccine strategies. In India, ART has been scaled up since 2004 and the number of patients taking the drugs is increasing each year (NACO, 2009). WHO recommends the surveillance of HIV 1 drug resistance in countries where ARV therapy is being scaled up. A molecular epidemiology and drug resistance survey among antiretroviral naïve patients was conducted in Mumbai in 2004, which showed the epidemic was predominantly caused by subtype C and a low level of primary resistance to 3TC, one of the important NRTI drug described in WHO recommendation (Deshpande *et al*., 2004). In resource poor settings, WHO recommends the public health approach for anti retroviral treatment programs, according to which patients are treated with a standard regimen of fixed dose regardless of individual drug resistance profile.

Continuous surveillance for monitoring the circulation of antiviral resistant strains among antiretroviral naïve patients is useful to alert ARV programs to make necessary adjustments, select effective drug regimen, and assess the effectiveness of HIV prevention programs. The study of molecular epidemiology will be useful to understand the dynamics of HIV- 1 epidemics, mainly epidemiological linkages and diversity of the strains circulating in certain geographic areas of the world.

HIV-1 has been characterized into four groups, M (Major), N (Non M non O), O (Outlier), and P. The group M has been reported to comprise about 95% of the global isolates and it has been divided into 9 subtypes, A, B, C, D, F, G, H, J and K, as well as many circulating recombinant forms (CRFs) (Plantier *et al.*, 2009, McCutchan, 2000). HIV-1 subtype C has been found to be most common in India whereas HIV-1 subtype B has been predominantly isolated from America, Australia and Europe. Circulating recombinant form CRF01_AE has been shown to be most prevalent in Thailand and Southeast Asia and Subtype G in Russia. Almost all subtypes have been reported from Sub-Saharan Africa but the subtypes A, C and D have been shown to be most prevalent (Schupubach, 2003). All of the Indian studies report the high prevalence of subtype C in major Indian cities. The prevalence of subtype C in North India has reported to be 78.4%, in Western India 96%, in Kolkata 95%, and 95% from South India. Other subtypes reported to be circulating in India are A and B (Ramalingan *et al.*, 2005, Sahani *et al.*, 2002, Mandal *et al.*, 2000, Gadkari *et al.*, 1998, Jameel *et al.*, 1995). Although about 95% of infections in India are of type C, the intersubtype recombinants between type A/C and B/C have been reported from Mumbai and northeastern India (Deshpande *et al*., 2004, Tripathy *et al.*, 2005).

Sequencing and phylogenetic analysis is the method of reference for drug resistance testing, subtyping and studying the genetic diversity of HIV- 1. In this method, plasma, or dried fluid spots (blood or plasma) can be used for amplification of whole or specific fragments of HIV- 1 genome. The amplified products are sequenced and the sequences are analyzed by bioinformatics softwares to study the subtypes, drug resistance and the genomic diversity among the isolates and other published sequences (Hongnes *et al.*, 2000). The samples in dried blood spots (DBS) and dried plasma spots (DPS) have been reported to be suitable for study of molecular surveillance, particularly in resource limited settings (Cassol *et al.*, 1996).

Currently more than 30 individual drugs and fixed dose combinations are available for treatment of HIV (Hammer *et al.*, 2008). Six different classes of antiretroviral drugs have been widely used for treatment of HIV 1; nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors and chemokine co-receptor antagonists. Antiretroviral treatment that uses the combination of three or more antiretroviral drugs from different classes is called as 'Highly Active Antiretroviral Therapy' (HAART). The HAART regimens generally consist of two nucleoside analogues and either protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (Yeni *et al.*, 2002).

HIV drug resistance is categorized as primary and secondary resistance. Primary resistance to antiretroviral therapy reflects the acquisition of drug resistant strain of HIV by newly infected person. HAART was initiated in 1996, and then resistance to single or multiple drugs have been reported from worldwide (Yerly *et al.*, 1999, Grant *et al.*, 2002). The type of resistance induced after a course of HIV treatment due to drug pressure is called secondary resistance. The induction of resistance to antiretroviral drugs is possible if mutant HIV quasispecies continue to replicate in the presence of level of drug that is insufficient to stop the viral replication but sufficient to exert a positive selective pressure on variants with decreased drug susceptibility (Clavel *et al.*, 2004). Mutations in reverse transcriptase and protease genes are associated with most of the drug resistance.

Drug resistance in HIV- 1 can be tested by genotypic or phenotypic methods. Genotypic methods use the genomic sequence data to detect the mutations that are associated with drug resistance. Usually, genotypic assays are used compared to phenotypic assays as they involve lower cost, are widely available, and have short turnaround time (Shafer, 2002). In phenotypic methods of susceptibility testing, a fixed inoculum of HIV-1 is cultured with different concentration of inhibitory drugs (Hertogs *et al.*, 1998). The rapidly increasing access to antiretroviral drugs has made HIV Drug Resistance (HIVDR) as a significant public health issue (Bertagnolio *et al.*, 2008). Regular population based assessment of transmission of drug resistant strains is important to assure continued efficiency of the limited ARV regimens in use in resource limited countries (Bennett *et al.*, 2008). The world health organization (WHO) has developed the HIV drug resistance threshold survey method for

surveillance of transmitted HIVDR in resource limited countries. Such survey should be conducted in geographical areas of country where large numbers of eligible patients are taking ARV for more than 3 years (Bennett *et al.*, 2008).

India discovered the first case of HIV in 1986 in Tamil Nadu (Simoes *et al*., 1987). The latest epidemic update estimation shows the total number people living with HIV in India to be 2.4 million (1.8-3.2 million) and the prevalence among the adults is 0.3% (0.3- 0.5%) (UNAIDS, 2008). Mumbai (formerly Bombay), the capital city of state of Maharastra in India is one of the high prevalence regions of HIV in India. The population of Maharastra is around 100 million with about 19 million people in Mumbai. The prevalence of HIV among the attendees of antenatal care clinics was about 0.5 % in 2007 (NACO, 2007). The epidemic of HIV in Maharastra is concentrated among Female Sex Workers (FSWs), Injecting Drug Users (IDUs) and Men having Sex with Men (MSM) (NACO, 2007).

The government of India freely provides first line ART services to people in need, since 2004. India has massively scaled up the first line ART service since 2007 although the coverage is still under 50% compared to those in need. As of March 2010 report of NACO, there are a total of 269 ART centers providing ARV services to 315,640 people living with HIV/AIDS in India. In Maharastra, there are 50 ART centers and 75,000 people are getting ARV services through these centers (NACO, 2009, NACO, 2010a).

This study aimed to survey the prevalence of drug resistance in antiretroviral naïve patients from Mumbai, India after 5 years of the massive scale up of antiretroviral drugs by government of India. The study also assessed the distribution of HIV -1 subtypes, polymorphisms and genetic diversity of the new isolates. The data are expected to be helpful to the public health authorities in Mumbai, India for managing effective antiretroviral therapy in ARV programs and the sequence data would be helpful for assessing the diversity and polymorphisms in Indian HIV -1 isolates.

CHAPTER II

2. OBJECTIVES

2.1 General objective

To study the molecular epidemiology and to survey the transmitted drug resistance in HIV-1 isolates from antiretroviral naïve people living with HIV- 1 recruited in 2009-2010, in Mumbai, India.

2.2 Specific objectives

To measure the prevalence of HIV 1 resistance to NRTIs, NNRTIs, and PIs.

To study the prevalence of HIV 1 subtypes and CRFs distribution.

To study the phylogenetic diversity of the isolates.

CHAPTER III

3. REVIEW OF LITERATURES

3.1 Human Immunodeficiency Virus

HIV-1 discovered in 1983 (Barre-Sinoussi *et al.*, 1983), has been shown to be virologically and serologically associated with early and late stages of acquired immunodeficiency syndrome (AIDS) (Gallo *et al.*, 1984). HIV 1 is the virus responsible to global pandemic of AIDS (Schupbach, 2003). HIV-2 discovered in 1986 (Clavel *et al.*, 1986), is less pathogenic compared to HIV 1 (Schim van der Loeff *et al.*, 1999). 'AIDS is the end stage of virus mediated protracted pathogenic process in which the immune system of an infected person and its ability to control infections or malignant progressive disorders are progressively destroyed' (Schupbach, 2003). AIDS was first described in United States in 1981 among homosexuals with a sudden outbreak of opportunistic infections, *Pneumocystis carinii* pneumonia and Kaposi's sarcoma (Durack *et al.*, 1981, Gottlieb *et al.*, 1981). The HIV/AIDS epidemic has been a serious public health concern globally. With each year the gap between AIDS cases in developed countries and those in developing countries is growing ever wider. As patients in developed countries have access to advanced healthcare and access to HAART, developing countries struggle with minimal resources that can neither prevent the dramatic increase in AIDS- related deaths or prevent the new infections (Palella *et al.*, 1998).

3.2 Global HIV situation

The number of people living with HIV in 2008 has been reported to be 33.4 million (31.1-35.8 million) (UNAIDS, 2009).A total of 2.7 (2.4-3.0) million people were newly infected and 2 (1.7-2.4) million AIDS related deaths occurred in 2008. UNAIDS claimed that the continuous rise in number of people living with HIV is due to the effect of regular new infections in high numbers and beneficial impact of ARV Therapy. As the end of 2008, about 4 million patients in low or middle income countries are under ARV therapy, which a 10 times increase of last five years (UNAIDS, 2009).

3.3 HIV situation in India

The first case of HIV in India was reported in 1986 (Simoes *et al.*, 1987).The UNAIDS update in 2008 estimated the number of people living with HIV in india is 2,400,000 with the adult HIV prevalence of 0.3%. The epidemic of HIV in India is concentrated among high risk groups (UNAIDS, 2009, NACO 2010).

Source: www.avert.org

Figure 3.3: States of India worst affected by HIV 1 epidemic.

India started first national AIDS control program in 1987 to respond to the epidemic by conducting surveillance, screening of donated blood, and strengthening health education (NACO, 2007). In 1992, the government of India established national AIDS control organization (NACO) to regulate the policies, prevention and Control programs concerning HIV and AIDS in India (NACO, 2007). The HIV prevalence in some hard hit Indian states is shown in table 3.3.

Table 3.3 Prevalence of HIV in different states of India.

SN	State	Location	HIV prevalence	Reference
1	Andhra Pradesh	Southeast India	Antenatal Clinics (1%). STD clinics (17%) MSM (17%), FSWs (9.7%), IDUs (3.7%).	NACO (2007)
2	Goa	Southwest India	Antenatal Clinics (0.18%), STD Clincis (5.6%)	NACO (2007)
3	Karnataka	Southwest India	Attendees of Antenatal Clinics (0.5%), FSWs(5%), MSMs (17.6%).	NACO (2007)
4	Maharastra	West India	Antenatal Clinics (0.5%), FSWs (18%), IDUs (24%), MSMs (12%)	NACO (2007)
5	Tamil Nadu	South India	Antenatal Clinics (0.25%), IDUs (16.8%), MSM (6.6%), FSWs(4.68%)	NACO (2007)
6	Manipur	Northeast India	IDUs (17.9-32%), Antenatal clinics (0.75%), Estimated Adult HIV prevalence (1.57%)	NACO(2007). Mahanta *et al*. (2008)
7	Nagaland	Northeast India	IDUs (1.91%), Antenatal clinics (0.6%), STD Clinics (3.42%)	NACO (2007)
8	Punjab	Northern mainland India	IDUs (13.8%)	NACO (2008)

3.4 HIV classification

The two distinct types of AIDS viruses, HIV-1 and HIV-2 are the member of the genus Lentivirinae of the Retroviridae family. They are single stranded, positive sense, enveloped, icosahedral RNA viruses. The rapid mutation and recombination of the HIV genome, has led to the development of various distinctive clades or subtypes (Schupubach, 2003). Based on phylogenetic analysis, HIV-1 has been characterized into four groups; M (Major), N (Non M non O), O (Outlier) and a new P group. The group M has been reported to comprise about 95% of the global isolates and it has been divided into 9 subtypes, viz. A, B, C, D, F, G, H, J and K, as well as many circulating recombinant forms (CRFs) (Plantier *et al*., 2009, McCutchan, 2000).

3.5 Significance of Molecular epidemiology/genotype determination

3.5.1 Pathogenicity and Transmissibility.

It has been suggested that high genetic variability of HIV 1 can lead to increased viral fitness and possibly affect the virus transmissibility and pathogenicity. However, the effect of single factor such as subtype and its association with pathogenicity and transmissibility is difficult to confirm by studies, as the consequences may be affected by other factors.

A study from Tanzania suggests that subtype might have role in vertical transmission, with subtypes A and C, and recombinant viruses being more likely to be perinatally transmitted than subtype D (Renjifo *et al.*, 2001). However, study from Thailand shows no difference between subtype B and E and pathogenicity (Amornkul *et al.*, 1999). A study from SouthAfrica shows subtype B is associated with male homosexual transmission and subtype C with heterosexual transmission (van Harmelen *et al.*, 1997).

Regarding disease progression, a study from Senegal shows that the infection with subtypes C, D and G has been associated with eight fold faster disease progression than with subtype A (Kanki *et al.*, 1999). In another Ugandan study, subtype D was associated with faster disease progression and death than subtype A (Kaleebu *et al.*, 2002). Many researches also report that rapid progression to AIDS is associated with areas where non B subtypes are prevalent than in areas with high prevalence of subtype B (Mehendale *et al.*, 2002, Tatt *et al.*, 2001). Similarly, a study from Kenya

showed that infection with subtype D was associated with higher mortality rate and faster decline in CD4 count compared to patients infected with subtype A or C (Baeten *et al.*, 2007). However, according to a Swedish study with subtype A, B, C or D infection, disease progression was not associated with subtypes or ethnic group (Alaeus *et al.*, 1999). Similarly, another study in Western and West- Central Africa show a similar survival or clinical disease progression in patients with CRF02_AG infection compared to other CRFS or subtypes (Laurent *et al.*, 2002).

3.5.2 Antiviral resistance and response to therapy

HIV 2 and HIV 1 group O, exhibit intrinsic resistance to nonnucleoside reverse transcriptase inhibitors (Tuaillon *et al.*, 2004, Descamps *et al.*, 1997). However, most of the studies suggest that there is no association between the HIV 1 subtypes and outcome of antiretroviral therapy. In a French Cohort study, with 24% participants being infected with non B subtypes, the response to antiretroviral therapy in terms of clinical progression, CD4+ count and viral load was not associated with subtypes (Bocket *et al.*, 2005). Similarly, another study in patients of African origin in London also reports no significant difference in outcome of therapy in patients infected with subtype A, C, or D (Frater *et al.*, 2001). In a comprehensive review, Taylor *et al.* (2008) suggest that HIV 1 subtype have no effect to antiretroviral therapy, rather the polymorphisms found in different subtypes before the initiation of antiretroviral therapy may affect the genetic pathway of resistance (Taylor *et al.*, 2008).

Several studies report that protease sequences of non B subtypes in antiretroviral naïve patients' exhibit amino acid substitutions like K20R, M36I and H69K/Q which are reported to cause secondary resistance in subtype B (Holguin *et al.*, 2004, Grossman *et al.*, 2001).

The development of secondary resistance in response to ART might be associated with infection with particular subtype. In a study among patients without a prior treatment with didanosine or stavudine based regimen, infected with subtype B or C, K65R mutation emerged in subtype C infection within 8 months more rapidly than in cases of subtype B infection (Frarter *et al.*, 2001). In addition to K65R mutations, in subtype C viruses resistance to NNRTIs may develop through K103N, or V106M mutations, however in subtype B virus V106N mutation rarely develops (Grossman *et al.*, 2004).

3.5.3 Development of Vaccine

The development of effective, preventive HIV vaccine is the key to ultimate control of the pandemic (Johnston and Fauci, 2007). HIV 1, due to its genetic diversity and continuous evolution of viral population within an individual, shows the mutational escape from response by CD8 cytolytic cells, and neutralizing antibodies during the course of time (Goulder *et al.*, 2001, Kwong *et al.*, 2002). The key to the development of broadly effective HIV 1 preventive vaccine should utilize the ways to elicit broadly neutralizing antibodies, and generate strong cell mediated immune response (Girard *et al.*, 2006, Douek *et al.*, 2006). The use of few isolates to prepare vaccine would not be preventive for other subtypes or variants within the same subtypes. In order to develop a fully effective preventive vaccine many strategies are employed like, the use of consensus sequence, combination of immunogens from different subtypes, preparation of mosaic immunogens from computational optimization (Robertson *et al.*, 2008). So, understanding of molecular epidemiology and HIV genetic diversity is one of the key to the development of effective vaccine for prevention of HIV.

3.6 Geographical distribution of HIV 1 genotypes

3.6.1 Global distribution of HIV 1

HIV 1 is distributed all over the world, exhibiting a complex molecular epidemiology. All HIV 1 subtypes are prevalent in Africa. Group M is distributed all over the continents, whereas group O and N are geographically restricted to central Africa (Tatt *et al.*, 2001, Vidal *et al.*, 2000). Subtype C and circulating recombinant forms containing at least *env* gene of subtype C has been reported to account for 50% or more of all HIV 1 infection worldwide (Hemelaar *et al.*, 2006, Essex, 1999), whereas global prevalence of other subtypes like A, B, G, and D were 12, 10, 6, and 3% respectively. The remaining subtypes (F, H, J, K) together constituted less than 1% global distribution. Circulating recombinant forms, CRF01_AE and CRF02_AG, each accounted for 5% of global infection and remaining 8% infections were caused by other recombinant forms (Hemaleer *et al.*, 2006).

The most prevalent subtype in South Africa is C, in West Africa is A and in East Africa subtype A and D are most prevalent. However, in West Africa and in some parts of Central Africa, the most prevalent genetic form is CRF02_AG. In Western

and Central Europe, in Americas and Australia, the subtype B is most prevalent. In Indian subcontinent subtype C is highly prevalent, whereas in Southeast Asia CRF01_AE and subtype B are most prevalent (Thomson *et al.*, 2002). The newly emerged CRF35_AD has contributed to the HIV 1 epidemic of Afghanistan and in Iran particulary among the injection drug users (Naderi *et al.*, 2006, Sanders-Buell *et al.*, 2007).

3.6.2 Distribution of HIV 1 genotypes in Asia

All of the Indian studies report the high prevalence of subtype C in major Indian cities. The prevalence of subtype C in North India has reported to be 78.4%, in Western India 96%, in Kolkata 95%, and 95% from South India. Other subtypes reported to be circulating in India are A and B (Ramalingan *et al.*, 2005, Sahani *et al.*, 2002, Mandal *et al.*, 2000, Gadkari *et al.*, 1998, Jameel *et al.*, 1995). Although about 97% of infections in India are of type C, the intersubtype recombinants between type A/C and B/C have been reported from northeastern India (Deshpande *et al.*, 2004, Tripathy *et al.*, 2005).

Studies report that molecular epidemiology of HIV 1 is changing in different geographical areas. A study reported the primary segregation of subtype B among IDUs and CRF01_AE among heterosexuals, which later become uniformly mixed in population of Thailand, which gives the shape to present day epidemic (Tovanabutra *et al.*, 2004. In Asia, Central Myanmar and Yunan Province of China have been described as the HIV 1 geographic 'recombination hotspots' (Takabe *et al.*, 2003, Yang *et al.*, 2002).

In China about 54.2% infections are due to subtype B, 18.3% with CRF08_BC, 13% with CRF01_AE, 5.5% with CRF07_BC and 3.3% with subtype C (Katherine *et al.*, 2007). The CRF07_BC is mainly prevalent in IDUs of Northwest China and CRF08_BC is mainly prevalent in Southeast China (Su *et al.*, 2000, Piyasirisilp *et al.*, 2000). In Taiwan 44.7% infection are with subtype B, 53.4% with subtype CRF07_BC, and 1.5% with CRF01_AE, however among IDUs 98% infection were with CRF07_BC (Lin *et al.*, 2007). However, in Japan 81% of epidemic is contributed by subtype B (Hemelaar *et al.*, 2006).

An extensive subtype distribution of HIV 1 subtypes in Asia based on the data from the Los Alamos HIV database has been reviewed by Katherine *et al.* (2007) (Table 3.6.2).

Table 3.6.2: The geographic distribution of HIV 1 subtypes and CRFs in Asia, based on the information from the Los Alamos HIV sequence database.

Country	HIV-1 Subtype/CRF	Subtype distribution* (%)
Bangladesh	C	62.9
	A	11.4
	01_AE	8.6
	U	5.7
	G	5.7
	B	5.7
Cambodia	01_AE	97.2
	B	1.7
	Other	1.1
China	B	54.2
	08_BC	18.3
	01_AE	13.0
	07_BC	5.5
	C	3.3
	BC	3.0
	Other	2.8
Hong Kong	01_AE	100.0
India	C	91.7
	B	5.5
	A	1.8
	Others	0.9
Indonesia	01_AE	75.0
	B	25.0
Japan	B	78.9
	01_AE	20.1
	Other	1.0
Malaysia	01_AE	60.7
	B	18.0
	01B	17.4
	U	1.2
	33_01B	1.2
	Other	1.5
Myanmar	B	46.9
	01_AE	19.6
	01B	10.2
	01C	8.6
Myanmar (cont.)	C	6.5
	BC	5.7
	01BC	2.0
	Other	0.4
Nepal	C	100.0
North Korea	G	100.0
Philippines	B	75.4
	01_AE	12.3
	A	7.0
	C	3.5
Philippines	B	75.4
	01_AE	12.3
	A	7.0
	C	3.5
	D	1.8
Singapore	01_AE	66.8
	B	31.3
	C	1.5
	Other	0.4
South Korea	B	90.4
	02_AG	2.5
	A	1.4
	D	1.3
	01_AE	1.3
	Other	3.1
Taiwan	B	35.6
	07_BC	34.7
	01_AE	15.2
	G	6.6
	C	3.5
	A	2.1
	Other	2.3
Thailand	01_AE	89.7
	B	8.2
	01B	1.0
	Other	1.1

Source: Katherine *et al.*, 2007.

3.7 Methods for HIV 1 genotyping

3.7.1 Sequencing and phylogenetic analysis.

Sequencing and phylogenetic analysis is the reference method for subtyping and studying the genetic diversity of HIV 1. However, it is costly and labor intensive. In this method, Plasma or Dried fluid spots (Blood or plasma) can be used for amplification of whole or specific fragments of HIV 1 genome. The amplified products are sequenced and the sequences are analyzed by bioinformatics softwares to study the subtypes, drug resistance and the genomic diversity among the isolates and other published sequences (Hongnes *et al.*, 2000).

The samples in dried blood spots (DBS) has been reported to be suitable for study of molecular surveillance, particularly in resource limited settings (Cassol *et al.*, 1996). DBS are already extensively used in many resource limited settings as the specimen type for the HIV serosurveillance. DBS can also be made from anti-coagulated blood drawn for the purpose of CD4 cell enumeration or other routine clinical tests. In addition, special absorbent paper for blood collection are economical and commercially available (Bertagnolio *et al.*, 2008).

3.7.2 Heteroduplex mobility assay (HMA) subtyping.

HMA can be used for subtyping of HIV 1 isolates without sequencing the genome. The principle of HMA is based on the difference in electrophoretic mobility of a heteroduplex formed between the amplified PCR product of a sample and reference strain for the identification of genotypes (Delwart *et al.*, 1995). In the method of HMA to subtype HIV 1, the env and/or gag gene fragments are amplified and analyzed. This process is less reliable for subtyping in areas where multiple subtypes co circulate. However, the env gp41 based HMA has been reported to be useful for monitoring subtypes in areas with divergent strains of HIV 1 (Agwale *et al.*, 2001). The HMA subtyping might be a less costly and less sophisticated alternative to sequencing, particularly in resource limited areas where one or two subtypes circulate in the population.

3.7.3 V3 serological subtyping.

This is the simple laboratory procedure to subtype HIV 1. This method is based on the principle of binding of serum antibodies to peptides from the V3 loop of the envelope

protein from different subtypes (Taechowisan *et al.*, 1996). This method is not considered to be reliable in subtyping where multiple subtypes co circulate and it is not reliable to differentiate the subtype C and A (Murphy *et al.*, 1999).

3.8 Antiretroviral drugs and HAART

Currently more than 30 individual drugs and fixed dose combinations are available for treatment of HIV (Hammer *et al.*, 2008). Six different classes of antiretroviral drugs have been widely used to treat HIV. Nucleoside and nucleotide analogues, work by inhibiting the reverse transcription of the viral RNA genome into DNA. Non nucleoside reverse transcriptase inhibitors bind and inhibit reverse transcriptase, thus halting the reverse transcription of viral RNA into DNA. Protease inhibitors inhibit the function of protease which cleaves the precursor proteins, permitting the final assembly of the virus particles. Fusion inhibitors block the penetration of HIV virions in to the target cells. Integrase inhibitor work by binding to the viral enzyme integrase and inhibit the formation of provirus in human chromosomes. Chemokine coreceptor antagonist (either CCR5 or CXCR4) bind to co receptor on CD4 cells and block the viral entry (HIV InSite, 2010). Current drug regimens can prolong the survival of patient but not cure HIV. The common treatment for HIV 1 is to use combination of three or more drugs which include drugs from different classes. This is called HAART (Highly Active Antiretroviral Therapy). The HAART regimens generally consist three antiretroviral drugs, usually two nucleoside analogues and either protease inhibitor or a nonnucleoside reverse transcriptase inhibitors (Yeni *et al.*, 2002).

3.9 ART use and coverage in India

The introduction of Highly Active Antiretroviral therapy (HAART) and its efficient use has greatly reduced the morbidity and mortality associated with HIV 1 infection (Jaggy *et al*., 2003, Palella *et al.*, 1998). The government of India freely provides first line ART services to people in need, since 2004. India has massively scaled up the first line ART service since 2007 although the coverage is still under 50% compared to those in need. As of March 2010 report of NACO, there are a total of 269 ART centers providing ARV services to 315, 640 people living with HIV/AIDS. In Maharastra, there are 50 ART centers and 75,000 people are getting ARV services through these centers (NACO, 2009, NACO, 2010).

Although the government of India rolled out second line ARVs from 2008, the coverage is still very much low. As of the end of December 2009, about 750 peoples out of 3000 in need were getting the second line treatment (NACO, 2010).

3.10 HIV Drug Resistance: Development and mechanisms of resistance

3.10.1 Primary resistance

Primary resistance to antiretroviral therapy reflects the acquisition of drug resistant strain of HIV by newly infected person. Primary resistance due to the transmission of drug resistant mutants has been considered as a threat for long term efficacy of ARV therapy. Mutants exhibiting resistance to Zidovudine were described as early as 1992, after six years of introduction of this drug (Keelam *et al.*, 1992). HAART was initiated in 1996, and then resistance to single or multiple drugs have been reported from worldwide (Yerly *et al.*, 1999, Grant *et al.*, 2002). Prevalence of primary HIV resistance differs in different geographical regions, but researches report the increasing trend from most areas (Grant *et al.*, 2002). The prevalence of transmitted drug resistance is usually higher in areas where antiretroviral drugs were available from long time. In North America (Weinstock *et al.*, 2004, Novak *et al.*, 2005, Jayaraman *et al.*, 2006, Truong *et al.*, 2006) and Western Europe (Wensing *et al.*, 2005, Oette *et al.*, 2006, Cane *et al.*, 2005, Paraskevis *et al.*, 2005) the prevalence estimates of transmitted drug resistant HIV is usually higher (8-18% and 2-14%, respectively).

Apart from transmission of drug resistant HIV strains, some HIV 1 isolates are naturally resistant to some antiretroviral drugs. Studies have reported that some HIV 1 subtypes can be less susceptible to nonnucleoside reverse transcriptase inhibitors and protease inhibitors when compared with subtype B strains (Shafer *et al.*, 1997, Palmer *et al.*, 1998, Descamps *et al.*, 1998).

3.10.2 Secondary or acquired resistance

This is the type of resistance induced after a course of HIV treatment. The infection rate to new cells in an HIV infected individual is very high (Perelson *et al.*, 1996, Wei *et al.*, 1995). The reverse transcriptase of HIV causes on average one mutation for each viral RNA transcribed to DNA, thus making the process highly prone to error (Preston *et al.*, 1998, Roberts *et al.*, 1998). The extremely high number of HIV

particles produced per day, combined with erroneous multiplication of the virus, results in a complex and diverse mixture of viral population in an infected individual. Such viral population is called quasispecies, which differ from each other by at least one mutation. Any of the mutation, if can confer some selective advantage to the virus, like decrease in its susceptibility to an antiretroviral agent, the corresponding quasispecies will overtake the others. The induction of resistance to antiretroviral drugs is possible if mutant HIV quasispecies continue to replicate in the presence of level of drug that is insufficient to stop the viral replication but sufficient to exert a positive selective pressure on variants with decreased drug susceptibility (Clavel *et al.*, 2004).

3.11 Mechanisms contributing to drug resistance

3.11.1 Resistance to Nucleoside and Nucleotide analogues

Nucleoside and nucleotide analogues can mimic the natural deoxyribose nucleoside/nucleotide, however lack the 3' hydroxyl group. After incorporation of these analogues in viral DNA, further elongation of DNA chain can not occur because further incorporation of deoxyribonucleotide triphosphates (dNTPs) require free 3' hydroxyl group. Two mechanisms either the impairment in analogue incorporation and removal of the analogue from the prematurely terminated DNA chain, can contribute to resistance.

Impairment in analogue incorporation

Mutations in gene coding for reverse transcriptase can lead to the formation of slightly changed reverse transcriptase with impaired ability to incorporate the analogues in the growing chain of DNA. Major mutations for such type of resistance are M184V, Q151M complex mutation and K65R mutations. M184V mutation is the main mutation causing resistance to Lamivudine, by causing steric hinderance and induces very high level of resistance (Sarafianos *et al.*, 1999, Schuurman *et al.*, 1995). In this mutation, the amino acid methionine located at the 184 amino acid position in the center of the reverse transcriptase, is substituted by Valine, so that the lamivudine triphosphate can not be aligned for DNA chain synthesis due to steric hinderance (Sarafianos *et al.*, 1999). The Q151 M complex of mutation is usually seen in HIV 2. In this mutation, there is the change in the amino acid near to the nucleotide binding

site of the reverse transcriptase. This resistance develops during the course of the failure regimen containing stavudine and didanosine (Kosalaraksa *et al.*, 1999). The K65R mutation is induced usually in whom therapy with nucleoside or nucleotide analogues fail. This mutation can cause resistance to most analogues with the exception of Zidovudine (Clavel *et al.*, 2004).

Repair of the terminated DNA chain

The group of mutations, commonly termed as thymidine analogue mutations (TAMs), confer resistance by enhancing ATP or pyrophosphate- mediated removal of nucleoside analogues from the 3' end of the terminated DNA chain. In lymphocytes, ATP and pyrophosphate can attack the phosphodiester bond, casing the removal of the analogue (Meyer *et al.*, 1999, Arion *et al.*, 1998).

3.11.2 Resistance to nonnucloside reverse transcriptase inhibitors (NNRTIs).

NNRTIs bind to hydrophobic pocket, close to the catalytic site of reverse transcriptase, affecting the flexibility of the enzyme and impairing its ability to synthesize the DNA (Esnouf *et al.*, 1997). Mutations causing resistance to NNRTIs are associated with change in the amino acid structure of the targeted pockets by specific drugs in the reverse transcriptase, ultimately causing the reduction in affinity with the drug (Richman *et al.*, 1994, Boyer *et al.*, 1993). Resistance to NNRTIs is associated with mutations like Y181C, K103N, Y188C, Y188L etc (Bennet *et al.*, 2009).

3.11.3 Resistance to Protease Inhibitors.

In HIV, protease cleaves the precursor proteins specifically, releasing structural proteins and enzymes, critical for production of infectious virions. Protease inhibitors are the chemically synthesized molecules that mimic the structure of natural substrate of HIV protease. Such molecules have the strong affinity to bind with the catalytic site of the enzyme, thus inhibit the catalytic activity of the protease (Roberts *et al.*, 1990, Erickson *et al.*, 1994). Mutations causing amino acid substitution in the catalytic center of the enzyme or in distant sites can result in resistance to protease inhibitors (Kaplan *et al.*, 1994, Condra *et al.*, 1995). Mutations like M46I, M46L, L90M, D30N etc are associated with resistance to protease inhibitor (Bennet *et al.*, 2009).

3.11.4 Resistance to Fusion inhibitor.

HIV 1 penetrates the target cells by the interaction of viral envelope glycoprotein complex (gp 120- gp41) with the cell surface receptors. The gp41 is the fusogenic component of the complex. The fusion inhibitor, enfuvirtide (a 36 amino acid peptide) specifically binds with a portion of gp 41, thus blocking the fusion of virus with the host cell. The resistance to enfuvirtide usually develops due to mutation in the specific region of the gp41, called as HR1 (Rimsky *et al.*, 1998, Wei *et al.*, 2002).

3.12 Methods to test drug resistance

3.12.1 Genotypic method

Genotypic methods use the genomic sequence data to detect the mutations that is associated with drug resistance. Genotypic assays are widely used compared to phenotypic assays as genotypic tests have low cost, are widely available, and have short turnaround time. The genotypic methods can use HIV 1 RNA from the plasma or HIV 1 proviral DNA extracted from PBMCs. The extracted DNA or RNA is amplified in the portion of protease and reverse transcriptase genes to generate sufficient DNA for nucleotide sequencing (Shafer, 2002).

The HIV 1 RNA extracted from plasma represent the most currently circulating quasispcies in the infected individual because the half life of the virus in plasma is about 6 hours (Perelson *et al.*, 1996). However, the proviral PBMC (Peripheral Blood Mononuclear Cell) DNA may contain archive mutations also.

The standard method in HIV 1 genotyping is dideoxynucleotide sequencing. In this method new DNA chains are synthesized with PCR using mixture of dNTPs and dideoxynucleotide triphosphate chain terminators (ddNTPs). In such reaction, a new strand of DNA is synthesized until a ddNTP is added in place of the corresponding dNTP. Unlike the incorporation of dNTPs, the incorporation of ddNTPs is a chance event. So, such amplification reaction generates a set of DNA strands which are different in size to each other by only one nucleotide base. The strands can be separated using capillary polyacrylamide gel electrophoresis in automated sequencer which uses flurometric method to detect and interpret the nucleotide sequence.

Most of the laboratories use the in house methods for amplifying the viral genome, using reagents from separate vendors. However, genotyping kits like Trugene by

Visible Genetics, Canada and Viroseq by Applied biosystems, foster city, California are available (Dileanis *et al.*, 2000, Erali *et al.*, 2001). The reproducibility, in terms of interlaboratory sequence concordance, of the nucleotide sequence generated using HIV 1 DNA by the dideoxynucletide sequencing has been reported 99.7% and using HIV 1 RNA it is reported to be 99% (Demeter *et al.*, 1998, Shafer *et al.*, 2001).

3.12.2 Phenotypic method

In phenotypic assays of susceptibility testing, a fixed inoculum of HIV-1 is tested with different concentrations of drugs. In this method protease gene, RT gene and some of gag gene is amplified from HIV-1 RNA purified from patient sample. The amplified product is inserted into a pol-deleted recombinant virus to make a recombinant HIV-1. A standardized concentration of this virus is inoculated in a cell line, and virus multiplication is measured in different serial dilutions of the different antiretroviral drugs (Hertogs *et al.*, 1998, Petropoulous *et al.*, 2000).

3.13 WHO and HIVDR Survey

Regular population based assessment of transmission of drug resistant strains is important to assure continued efficiency of the limited ARV regimens in use in resource limited countries. The World Health Organization (WHO) HIV drug resistance (HIVDR) survey method is developed to survey HIVDR in newly infected antiretroviral naïve patients. Since, the problem of transmitted drug resistance would appear first in areas of world where ART has been widely used for many years and WHO recommends countries should begin surveillance in these areas (Bennett *et al.*, 2008).

3.14 HIVResNet Drug Resistance Laboratory Strategy

HIVResNet is a group of more than 50 international HIVDR experts, affiliated with universities, laboratories, international and non- profit organizations. The overall objective of the global strategy is to support country planning of ART programme practices, minimize the emergence of HIVDR, and to restrict the extent to which resistance jeopardizes the effectiveness of the standard first and second line recommended ART regimens available in resource limited settings. The molecular virology laboratory has the fundamental role in HIVDR surveys. According to this strategy, the global network of laboratories is organized on three levels: national drug

resistance laboratories (NDRLs), regional drug resistance laboratories (RDRLs), and global specialized drug resistance laboratories (SDRLs). The testing of specimens for HIVDR surveys should occur in WHO accreditated laboratories. The HIVResNet group has provided the recommendations for the collection handling, shipment, storage and testing of specimens (Bertagnolio *et al.*, 2008).

3.15 HIVDR Prevention and assessment strategy

The rapid scale up of antiretroviral therapy for HIV in resource limited setting is international priority with the goal of universal access by 2010 (UNAIDS, 2007). The rapidly increasing access to antiretroviral drugs has made HIVDR as a significant public health issue (Bertagnolio *et al.*, 2008). The WHO has been promoting the public health approach to antiretroviral therapy scale up in developing countries (Gilks *et al.*, 2006). So, WHO in association with HIVResNet group has developed a global HIV Drug Resistance (HIVDR) prevention and assessment strategy (Bennett *et al.*, 2008). WHO recommends the implementation of this strategy by National HIVDR working groups in countries scaling up ARV therapy.

3.16 Surveillance of transmitted HIV drug resistance

The world health organization (WHO) has developed the HIV drug resistance threshold survey method for surveillance of transmitted HIVDR in resource limited countries. Such surveillance is recommended for geographical areas of country where large number of eligible PLHAs are taking ART for more than 3 years. HIVDR survey requires only $\leq$ 47 specimens (Bennet *et al.*, 2008). The cost of the surveillance can be minimized using routine patient information and remnant blood or plasma samples. Similarly, eligibility criteria have been recommended so that ARV experienced patients and patients infected before wide availability of ART are excluded from the survey. The survey categorizes the drug resistance to each relevant drug class in threshold of <5%, 5- 15% or >15%. The result of survey has been expected to be useful in management of ART in country and to prevent the emergence and transmission of drug resistance (Bennett *et al.*, 2008).

CHAPTER IV

4. METHODOLOGY

4.1 Study site and Sample size.

The study population includes HIV 1 infected patients in Mumbai or suburbs of Mumbai, the Capital city of Maharastra, India. All the patients included in the study were antiretroviral naïve. A total of 68 blood samples were collected on consideration that at least 47 DNA sequences are needed to have interpretation of drug resistance results according to WHO drug resistance threshold survey (Bennet *et al.,* 2008).

4.2 Preparation, transport and storage of DPS.

Dry Plasma Spots were prepared using 50ul of plasma on at least two circle of Whatman 903 paper as described by Deshpande et al. (2010). The samples were transported at room temperature from Mumbai to Bordeaux. After arrival in Bordeaux, the samples were stored at -80^{o}C. All the laboratory processing for amplification and sequencing was done in Laboratorie de Virolgie, Université Victor Segalen, Bordeaux 2.

4.3 Elution of virions from DPS

This step was performed in a room reserved for the extraction of the nucleic acid to prevent the spread of genetic material that can contaminate other manipulations. The usual laboratory coat was removed and left outside the room. A clean coat, hung inside the room was used. Gloves were used in handling each specimen, and changed frequently to minimize contamination. The manipulations were done under a hood cleaned and disinfected by UV light.

- DPS were taken out of -80° C, and allowed to equilibrate to room temperature.
- Scissor and forceps were disinfected in ethanol bath, and were used to cut the DPS.
- Firstly the plasma spot was cut in circle, and then the spot was cut in 3 vertical bands of equal value and placed in the corresponding 1.5 ml tube.
- Same operation was repeated for other DPS samples, with disinfection of scissor and forceps in ethanol bath for each sample.

- In each tube containing the spots cut out, 220 µl of elution buffer was added.
- The tubes were vortexed for few seconds.
- All the tubes were rotated on a rotatory shaker at 4° C for 1hour.

4.4 Extraction **of viral RNA by QIAamp Viral RNA Mini Kit (Qiagen, Germany)**

- Lysis buffer / binding AVL (Buffer AVL+RNA carrier), wash buffer 1 (AW1) and wash buffer 2 (AW2) were prepared according Qiagen Viral RNA Minikit protocol.
- The tubes containing the elution of the spots were vortexed and then centrifuged for few seconds.
- Supernatant (140 ul) was pipetted and mixed with 560ul lysis buffer in another tube.
- Viral RNA was extracted, strictly following the Qiagen protocol.
- The RNA extract was stored at 4°C, before commencing for RT PCR (maximum 2 hours).

4.5 RT-PCR

i. Choice of primers

For the amplification of the reverse transcriptase gene in RT PCR, following set of primers was used.

RTin1: AGTATTAGTAGGACCCACACC

RTin2: TGCTTCTTCAGTTAGTGGTACT

For the amplification of the protease gene following set of primers were used:

PRin1: TTTTTTAGGGAAAATTTGGCCTTC

PRin2: ATTTTCAGGCCCAATTTTTGTAAT

Primers given in 5 'to 3'

ii. RT PCR mixture was prepared according to the kit protocol (InVitrogen,), in following proportion for 1 reaction.

- 2X Reaction Mixture 25 ul
- Primers 5’ 1 ul
- Primer 3’ 1ul
- Enzyme 1 ul
- Sterile water 12 ul

➢ In each PCR tubes 40 ul of reaction mixture was added.

➢ Ten microliter of viral RNA extract was added on each tube, except the negative control.

iii. RTPCR was run in programmable thermal cycler (Primus, Germany)

- 30’ at 50 ° C, 2’ at 94 ° C.
- [30’’ at 94° C (denaturation), 30’’ at 55 ° C (primer annealing) and 1’30’’ at 68° C (elongation)] for 40 cycles.
- 7’ at 68° C.
- Holding at 8 ° C

4.6 Nested PCR

The nested PCR is a conventional PCR amplification of DNA strands from the product obtained by RT-PCR. The primers must be specific but mostly internal to those used in RT-PCR so that they can hybridize with DNA fragments amplified by RT-PCR. The amplified fragment will be smaller than that obtained previously by RT PCR.

i. Choice of primers

For the amplification of reverse transcriptase gene:

RTin3 TTGGATGCACACTAAATTTTCCAA

RTin4 CTTGCCCAGTTTAATTTTCCCAC

For the amplification of the protease gene

PRin3 CAGACCAGAGCCAACAGCCCCA

PRin4 TCTTCTGTCAATGGCCATTGTTT (*Primers in 5 'to 3')*

ii. Nested PCR mixture was prepared according to the kit protocol (Amplitaq Gold, Applied Biosystems), in following proportion for 1 tube reaction.

- Buffer MgCl2 (25 mM) 10 ul
- 10X Buffer 10 ul
- Sterile DW 73.6 ul
- dNTPs (10 mM) 2 ul
- Primer 5'(20uM) 1 ul
- Primer 3'(20uM) 1 ul
- Taq Gold enzyme 0.4 ul

- PCR tubes were taken as needed and 98ul of reaction mixture was added in each well.
- Two microliter of RTPCR product was added in each well, except in the wells of negative control.

iii. Nested PCR was run in programmable thermal cycler (Primus, Germany) with the following program.

- 12' at 94 ° C.
- [30'' at 94° C (denaturation), 30'' at 55 ° C (primer annealing) and 2' at 72° C (elongation)] for 40 cycles.
- 7' at 72° C.
- Holding at 8 ° C

4.7 Agarose gel electrophoresis.

- Agarose gel electrophoresis was run to check the successful amplifications in nested PCR products.
- Agarose gel (1.5%) with Ethidium bromide was prepared in the gel moulder using appropriate combs.
- TBE solution (0.5%) was added to cover the gel.
- The nested PCR product (5 ul) was mixed with 6X DNA loading dye, and added to the respective well in the gel.
- Electrophoretic migration was done at 120 volt for 30 minutes.

- The DNA bands in the gels were revealed by using UV Transilluminator System, and images were printed.

4.8 Purification of amplicons.

- Column for purification was reconstituted by vortexing for 20 second according to S400 Roche protocol.
- The column was inversed, tip was broken and inserted into collection tube
- Centrifuged for 3 minutes at 2500 (Rotation per minute) RPM.
- The column was inserted into another DNA collection tube.
- All the volume of nested PCR product was added on the column.
- Centrifuged for 5 minutes at 2500 RPM.
- The DNA in the collection tube was secured.
- Purified nucleic acid was stored in -80 ^{o}C refrigerator

4.9 Sequencing reaction.

DNA sequencing reaction was performed by using Beckman Coulter DTCS (Dye Terminator Cycle Sequence) kit. The sequencing procedure was bi-directional, therefore the study applied separately the sense primer and anti-sense primers to generate both strand of reverse transcriptase and protease genes. The primer set used for sequencing reaction were the same as for nested PCR, but in lower concentration (3uM).

The operating procedure followed manufacturer's protocol. Following table shows the formulation of the DNA sequencing reaction mixture for 1 tube recation.

Reagents	Volume (ul)
• Primer	2.0
• Master Mix Solution	8.0
• Nested PCR product	10.0

Operation program setting of thermocycler followed the manufacturer's manual:

- [20'' at 96° C (denaturation), 20'' at 50 ° C (primer annealing) and 4' at 60° C (elongation)] for 30 cycles.

- Holding at 8 ° C.

4.10 Sequencing migration.

The sequencing migration was performed in an automated sequencer Beckman CEQ 2000 DNA Analyzer System.

- 300μL of separation buffer was added in each well of buffer plate (from the GenomeLab^{TM} DTCS kit)
- Sephedex gel was prepared in Millipore 96-well filter plate.
- Sequencing reaction product was added (20μL) to the respective wells of sephadex plate, then centrifuged at 2500 rpm for 5mins to collect the DNA in the Greiner 96-well plate.
- The DNA was mixed with formamide and transferred in to sample plate.
- Each well was covered with mineral oil.
- The buffer plate and sample were loaded in the sequencing machine for sequencing migration according to manufacturer protocol.

4.11 Sequence reading and result interpretation.

The sequences were read on both strands. Manual editing of the sequences was done in points of ambiguity by the software. The resistance mutations to NRTIs, NNRTIs and PIs were interpreted according to WHO mutation list for 2009, ANRS Algorithm and Stanford Algorithm (Bennet *et al*., 2009, http://www.hivfrenchresistance.org/, http://hivdb.stanford.edu/).

4.12 Subtyping and Phylogenetic analysis.

The RT and protease sequences were aligned by the software Clustal W 1.83 with known reference sequences of M, N and O group strains retrieved from Los Alamos Sequence Database (http://www.hiv.lanl.gov). Phylogenetic trees were inferred using the neighbor-joining method with a Kimura two-parameter algorithm with the software NJ plot. Subtyping was also confirmed by web based REGA HIV 1 subtyping tool version 2 (http://dbpartners.stanford.edu/RegaSubtyping/).

4.13 Ethical consideration

The study was reviewed by the ethical review committee of Sir JJ Hospital, Mumbai. Patient's samples were uniquely coded for the identity, and tested anonymously in the laboratory.

4.14 Data management.

Microsoft Excel sheet was used to manually align the amino acid sequences, of the isolates to look for the DRMs, and polymorphisms. The results were confirmed by SmartGene IDNS service module (https://apps.idns-smartgene.com/apps/IDNSPortal.po).

CHAPTER V

5. RESULTS

5. 1 Subtype distribution of HIV 1 isolates from Mumbai, India.

Among the total 68 samples, 52 samples could be amplified on reverse transcriptase and protease gene. Of these 52 amplified products 51 could be sequenced for reverse transcriptase gene and all of the 52 were sequenced for protease gene. In the study population, subtype C is found to be predominant. All of the 51 reverse transcriptase sequences clustered with subtype C sequences (Figure 5.3). Among the total 52 protease sequences, 51 sequences belonged to subtype C, whereas one of the isolate clustered with subtype A sequences (Figure 5.4). The subtyping was inferred from the phylogenetic tree, and was also confirmed by REGA HIV 1 subtyping tool. There was absolute concordance of results by both of the techniques. Although most of the sequences were of subtype C, we have confirmed the circulation of A-C intersubtype recombinants in Mumbai.

5. 2 Prevalence of resistance to antiretroviral drugs.

Resistance to antiretroviral drugs was observed to NNRTIs in position K103N, and V106M and to PIs in position M46I. All the three mutations are listed in WHO surveillance drug resistance mutation list (Bennet *et al*., 2009). Two of the isolates had V106M mutation, 1 isolate had K103N and V106M mutation, one had K103N as well as M46I mutation and one had only M46I mutation. So, the prevalence of resistance to drug class NNRTI was 7.8%, which is in the 5-15% threshold of WHO for surveillance of transmitted drug resistance. The prevalence of resistance to PIs (3.8%) was below the WHO threshold of 5%. The overall prevalence of drug resistance to NRTIs, NNRTIs, and PIs is about 10% (Table 5.2). No resistance mutation could be observed to the drug class NRTIs. All of the isolates exhibiting resistance belonged to subtype C on both reverse transcriptase and protease gene, according to the phylogenetic analysis.

Table 5.2 Prevalence of resistance mutation to different ARV drug class.

SN	Sample id	Resistance mutation	Resistance conferred to	Prevalence of resistance to
1	in1033	K103N, M46I	NNRTIs, PIs	NNRTIs = 7.8%(4/51) PIs=3.8% (2/52) Overall prevalence = 9.6%(5/52).
2	in1050	V106M	NNRTIs	
3	in1098	V106M	NNRTIs	
4	in1113	K103N, V106M	NNRTIs	
5	in1097	M46I	PIs	

5. 3 Phylogenetic diversity of Mumbai isolates in reverse transcriptase gene.

All of the isolates with reverse transcriptase sequences clustered with subtype C references with high bootstrap value. The subtype C references clustering with our isolates were Ref. C.ZA.04.SK164B1, Ref.C.ET.86.ETH320, Ref.BR92.BRO25_D. and Ref. C.IN.95.IN21068 (Fig 5.3).

5.4 Phylogenetic diversity of Mumbai isolates in protease gene.

Among the 52 protease sequences, 49 clustered with subtype C references and one sequence clustered with subtype A references. Two of the protease sequences were just outside the clusters of subtype C. The result indicates that the Indian isolates are more diverse in the region of protease than in reverse transcriptase (Fig 5.4).

5.5 Polymorphisms in reverse transcriptase gene.

The amino acid sequence deducted from the nucleotide sequences were aligned with subtype B reference sequence (HXB2) to find the polymorphisms from the codon 1 to 250. A total of 20 positions of polymorphism of more than 10% was noted. In fifteen positions, the polymorphism was more than 50%. In 9 positions, the percentage of polymorphism was more than 90%. Only a single type of amino acid substitution was observed in 7 positions. About 14% polymorphism was noted in codon 179, which is in the WHO list for surveillance drug resistance mutation (SDRM). Absolute polymorphism was noted in T39E/D/N, K173A/T/S/E/I and V245Q/L/H/E.

Table 5.5 Amino acid polymorphisms in specific positions of RT gene.

SN	Ref. Amino acid/ Position polymorphism	Observed amino acids	Sequences with polymorphism	Total Sequences	% Polymorphism
1	V/35	T/I/K/M/Q	50	51	98.04
2	E/36	A	27	51	52.94
3	T/39	E/D/N	51	51	100.00
4	S/48	T	49	51	96.08
5	V/60	I	48	51	94.12
6	D/121	Y/H	36	51	70.59
7	I/135	R/T/L/K/V/M	31	51	60.78
8	S/162	A/H/C/G/N/F/D	34	51	66.67
19	K/166	R	7	51	13.73
10	K/173	A/T/S/E/I	51	51	100.00
11	Q/174	K/R	16	51	31.37
12	D/177	E	43	51	84.31
13	I/178	L/M/V	15	51	29.41
14	V/179	D/E/I	7	51	13.73
15	T/200	A/E/I	50	51	98.04
16	I/202	V	6	51	11.76
17	Q/207	A/E/G/T/K/N	50	51	98.04
18	R/211	K	43	51	84.31
19	L/214	F/C/L	46	51	90.20
20	V/245	Q/L/H/E	51	51	100.00

5.6 Polymorphisms in protease gene.

The polymorphisms were noted as compared to the subtype B reference sequence (HXB2) from codon 1 to 100. A total of 15 positions of polymorphisms of more than 10% was observed. In nine positions, the polymorphism was more than 50%. In 5 positions, the percentage of polymorphism was more than 90%. Only a single type of amino acid substitution was observed in 9 positions. About 20% polymorphism was noted in codon 82, which is in the WHO list for surveillance drug resistance mutation (SDRM). Absolute polymorphism was noted in H69K/Q and I93L.

Table 5.6 Amino acid polymorphisms in specific positions of protease gene.

SN	Ref. Amino acid/ Position polymorphism	Observed amino acids	Sequences with polymorphism	Total sequences	% Polymorphism
1	T/12	S/A	41	52	78.85
2	K/14	R	15	52	28.85
3	I/15	V	36	52	69.23
4	G/16	E	11	52	21.15
5	L/19	I/V/T/M	48	52	92.31
6	K/20	R	8	52	15.38
7	M/36	I/V/L	49	52	94.23
8	N/37	D/S/C	12	52	23.08
9	R/41	K	43	52	82.69
10	D/60	E	4	52	7.69
11	Q/61	H/E	8	52	15.38
12	L/63	T/P/S/H/A/L/V/Q	49	52	94.23
13	H/69	K/Q	52	52	100.00
14	V/82	I	10	52	19.23
15	L/89	M	31	52	59.62
16	I/93	L	52	52	100.00

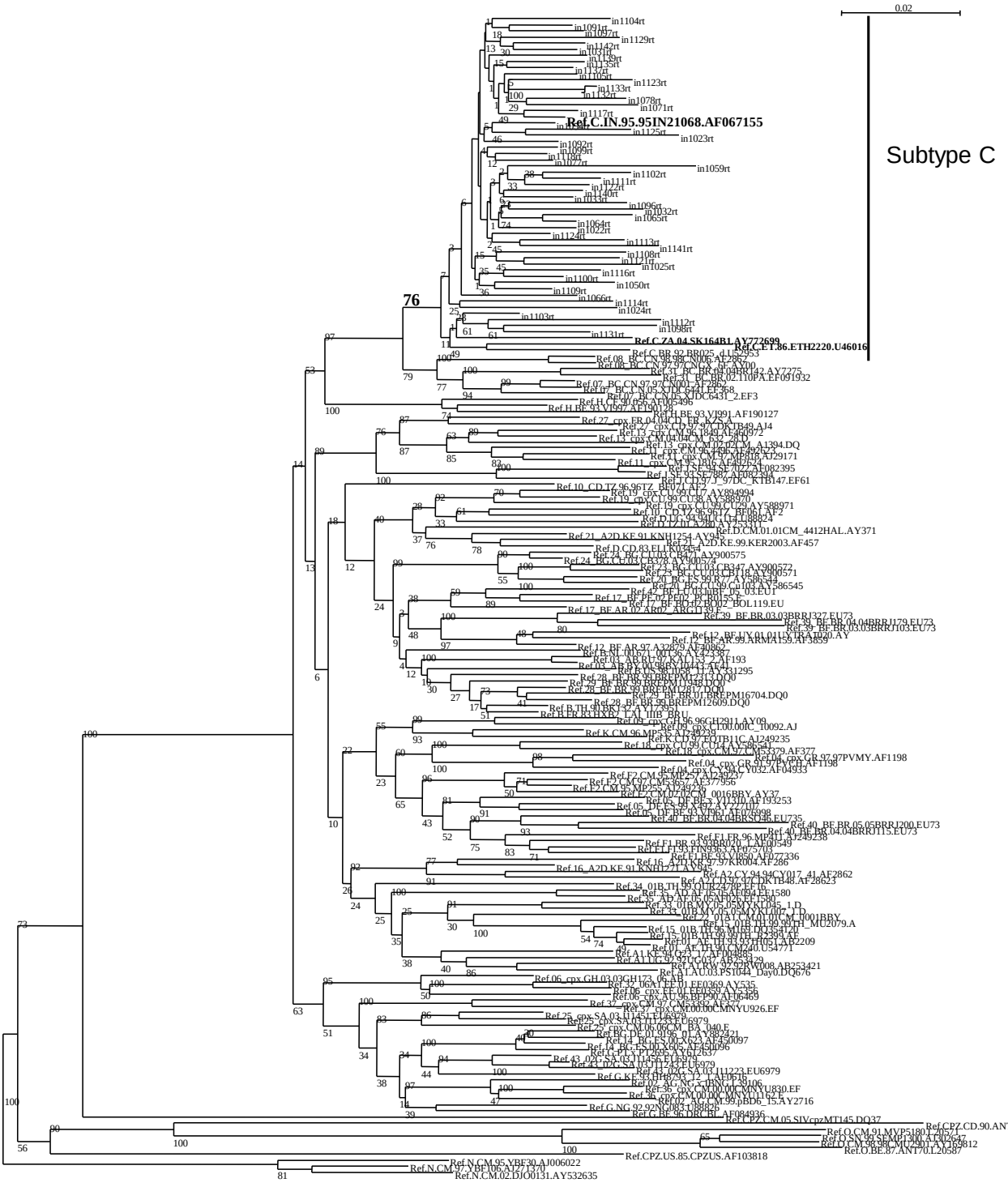

Figure 5.3 Phylogenetic Tree of sequences of reverse transcriptase gene. The group N was used as root. All of the isolates clustered with high bootstrap value to the subtype C reference sequences.

Figure 5.4 Phylogenetic Tree of sequences of protease gene.

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6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

The study shows predominance of subtype C as a major subtype responsible for HIV 1 epidemic in Mumbai, India, although there is evidence of A-C inter subtype recombinants circulating at low level. We demonstrated for the first time, the prevalence of resistance to antiretroviral drug classes to be around 10% in antiretroviral naïve patients from Mumbai. As well as, this is the first time there has been evidence of drug resistance towards PIs in Mumbai among the antiretroviral naïve population. High level of polymorphism was observed in many amino acid positions, both in reverse transcriptase and protease gene, compared to the subtype B reference (HXB2). Polymorphisms were also observed in amino acid positions, listed by WHO to be used as Surveillance Drug resistance Mutations (SDRMs).

The phylogenetic analysis of 51 reverse transcriptase gene sequence of present study, and 138 reference sequences from Los Alamos database (http://www.hiv.lanl.gov) showed, all the sequences clustered with pure subtype C reference sequences. This survey, once again confirmed very high prevalence of subtype C viruses in Mumbai which is in concordance with the study conducted on the similar population of same study site in 2004 (Deshpande *et al*., 2010, Deshpande *et al*., 2004). In present study, one isolate had its reverse transcriptase clustering with subtype C, but its protease sequence clustering with subtype A (A1), indicating a circulation of inter subtype recombinant in Mumbai. There had been evidences of low level circulation of A-C recombinants in Mumbai, other parts of southern India, and western India (Lall *et al*., 2008, Deshpande *et al*., 2004, Lole *et al*., 1999). Deshpande *et al*. (2004) have also demonstrated the presence of pure subtype A and CRF_01 AE, which cannot be observed in present study. Apart from predominance of subtype C in every part of India, there has been a relatively higher prevalence of B-C intersubtype recombinants in Northeastern part of India (Tripathy *et al*., 2005). However, the effect of subtypes on disease progression is controversial, the replicative fitness and transmission efficiency of Indian subtype C viruses is significantly higher over subtype A, and A-C recombinant clone, which might be a possible explanation of a sustained and higher

level prevalence of subtype C viruses all around India even though there is introduction of other subtypes since many years (Rodriguez *et al*., 2009).

The study shows a 7.8% prevalence of resistance to NNRTIs, which is in WHO threshold of 5-15% (moderate level resistance). The mutations K103N, and V106M are the drug resistance mutations listed in WHO list of SDRMs for surveillance of transmitted drug resistance. Both of these mutations are listed in French ANRS (http://www.hivfrenchresistance.org/) and Stanford University HIV 1 drug resistance database (http://hivdb.stanford.edu/). K103N mutation causes high level of resistance to all of available NNRTIs, and is able to cause virologic failure to each of NNRTIs during treatment (Bacheler *et al*., 2001, Joly *et al*., 2000). V106M mutation confers higher level resistance to all NNRTIs in subtype C viruses, compared to subtype B viruses (Brenner *et al*., 2003).

This is the first time that a moderately high level of drug resistance to NNRTI is observed in Mumbai, among the antiretroviral naïve population. A similar study in 2004, in the similar population of same study site did not note any resistance to NNRTIs. However, at that time the access to antiretroviral drugs in Mumbai, or other part of India was very limited. India scaled up the free HAART, to patients in need from 2004. The HAART regimen for the first line treatment in India, including Mumbai is, d4T/AZT-3TC-NVP/EFV (2 NRTIs+1NNRTI) (NACO, 2009). So, there have been increased chances of transmission of drug resistance to newly infected peoples in recent years. Higher level of drug resistance in antiretroviral naïve patients have been observed in areas of world where antiretroviral drugs are widely available and accessible from a longer time period (Novak *et al*., 2005, Jayaraman *et al*., 2006, Cane *et al*., 2005).

The first line HAART regimen in India contain two drug classes, and prevalence of resistance to one of the classes is observed to be moderately higher in this study, indicates that the chances of treatment failure would be higher and there is increased probability of further transmission of drug resistant viruses to newly infected people. This vicious cycle can ultimately lead to high prevalence of circulation of drug resistant HIV 1 viruses, limiting the availability of effective and affordable drugs. So, the public health authorities concerning HIV 1 treatment and prevention should plan further studies and careful observations of factors that might be leading to

transmission of drug resistance strains, and adjustment in their programs to minimize the emergence of drug resistance in future. The management of treatment for patients with primary resistance to ART, is relatively easier in economically developed world, due to accessibility to advanced healthcare system. However, management of treatment in context of higher level of primary resistance to antiretroviral is very difficult, in developing world where access to drug resistance testing and monitoring of success of HAART in terms of immunological and virological parameter is still limited. The ARV programs in developing world are based on the public health approach, in which patients are treated with a fixed regimen, irrespective of the individual susceptibility to antiretroviral drugs. HAART can be sub-optimally effective, in context of higher level of primary resistance, ultimately leading to failure of ARV programs in developing countries. So, an ongoing surveillance of DRMs in recently infected antiretroviral naïve population is important to monitor the prevalence of primary resistance, and to make timely adjustment in ARV programs.

Present study did not show any mutation conferring resistance to drug class NRTI. However, a low prevalence of M184V mutation, conferring resistance to NRTI, especially Lamivudine (3TC) was observed in similar population, in the same study site in Mumbai (Deshpande *et al*., 2004). M184V mutation has been showon to be the most frequent mutation to be developed in condition of ART failure in India (Deshpande *et al*., 2010, Gupta *et al*., 2010, Vidya *et al*., 2009, Kandathil *et al*., 2009). The M184V mutation can revert into wild type virus in absence of any drug pressure after transmission into new individuals (Brenner *et al*., 2002, Gandhi *et al*., 2003), which would not be detected by the population genome sequencing methods, like in this study. Such condition may lead to underestimation of the transmitted drug resistance. Other studies in India among antiretroviral naïve patients infected with subtype C viruses, have not noted any major drug resistance to NRTIs, NNRTIs and PIs (Arora *et al*., 2008, Balakrishnan *et al*., 2005, Sen *et al*., 2007).

This study shows a 3.8% (M46I) prevalence of resistance conferring to PIs. M46I mutation confers high level resistance to many PIs (Condra *et al*., 1996, Molla *et al*., 1996). Although the prevalence level observed to PIs is low, the appearance of resistance to PIs is very interesting as PIs are not included in the first line national HAART regimen in India, and neither in Mumbai. However, people undergoing in

private clinics for ARV might have access to these drug. Gupta *et al*. (2010) has shown that patients are taking PI based regimens in private clinics in Mumbai, and reported appearance of resistance to PIs in patients under treatment. Similar to this study, M46I mutation has been observed in antiretroviral naïve patients in North India (Arora *et al*., 2008). This is the first time that the resistance to PIs has been observed in Mumbai among antiretroviral naïve population.

The study noted key polymorphisms compared to subtype B reference HIV 1 virus. At least 10% polymorphism has been observed in 20 amino acid positions of reverse transcriptase gene, whereas >90% polymorphism was observed in 9 positions. About 14% polymorphism was observed at position V179, which is the position that can bear drug resistance mutation, and is listed in WHO SDRMs list. The V179D mutation was observed in 7.8% of isolates, which according to Stanford Algorithm of HIV 1 drug resistance interpretation is associated with potential low level resistance to NNRTIs. Compared to similar studies, a decrease in polymorphism was observed in position E36A, K166R, whereas the polymorphism pattern in other positions of reverse transcriptase gene was similar (Deshpande *et al*., 2010, Deshpande *et al*., 2004).

A high level of polymorphism was observed in protease gene at positions M36, and H69 which are listed as minor (secondary) resistance mutations for subtype B in ANRS algorithm. The position V82, is the position associated with DRMs, and about 20% polymorphism was observed in this point from the Indian HIV 1 isolates. Similar polymorphism pattern in protease gene was described earlier in Mumbai, and other cities in South India (Deshpande *et al*., 2004, Kandathil *et al*., 2009). Although the importance of polymorphisms is not studied extensively, and is difficult to establish, it might be associated with virus fitness, and replicative capacity. The profile of polymorphism, listed in this study would be useful for similar future studies to understand the evolution and dynamic nature of HIV 1in different geographical regions of the world.

This study has demonstrated a crucial situation of HIV 1 drug resistance level in antiretroviral naïve patients in Mumbai, however the study has some limitations. The seroconversion date of patients was not known so it is not clear whether the transmission occurred after scaling up of free ARV service in India, or before ? About

20% of HIV positive samples from DPS could not be amplified, and sequenced. If these samples contain DRMs, the prevalence in the study might be underestimated and vice versa. All of the patients come from Mumbai, however it is not clear that in which geographical region of the country, they acquired the infection?

6.2 CONCLUSION

The HIV 1 epidemic in Mumbai is predominantly caused by subtype C viruses, phylogenetically more related to African subtype C viruses, although there is evidence of low level circulation of A-C intersubtype recombinant viruses. The prevalence of drug resistance observed to be around 10% (WHO threshold, 5-15%) urges the ARV program authorities in Mumbai to take timely preventive actions for minimizing the emergence and transmission of drug resistant HIV 1viruses and to study for possible adjustment in programs to prevent treatment failure in first line HAART. Substantial polymorphism is observed compared to subtype B reference virus, however the polymorphism pattern in HIV 1 isolates from Mumbai seems to be stable when compared to previous studies.

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