STUDIES ON MOLECULAR DIVERSITY OF HUMAN IMMUNODEFICIENCY VIRUS-1

THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (SCIENCE)

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BY

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled ***"Studies on Molecular Diversity of Human Immunodeficiency Virus-1"*** submitted by Smt. **Srijita Nandi** who got her name registered on **29-10-2015** with **Registration Index No.: 220/15/Life Sc./24** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Malay Kumar Saha, Ex. Scientist F, Department of Virology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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(Signature of the Supervisor date with official seal)

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Dedicated To

My beloved Parents

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Srijita Nandi.

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ABSTRACT

Index No.: 220/15/Life Sc./24

Title: Studies on Molecular Diversity of Human Immunodeficiency Virus -1

India having the third major HIV epidemic in the earth with an estimation of 2.1 million people is living with HIV and more than 65 thousand deaths in past years. Transmission of HIV with anti-retroviral drug resistance mutations has a negative impact on prevention of HIV through ART. Studies on genetic diversity of HIV are hardly reported in India. There are limited data available on drug resistance mutation in treatment naïve patients, treatment failure on first line ART in resource limited settings, DRM in second line ART failure patients as well as on virological failure. Evidence on subtype diversity and drug resistance testing of infants (Age 6 weeks to 18 months) born to HIV positive mothers are also very rare.

This study aims to find out circulating subtypes, drug resistance mutations and phylogeny diversity of HIV from different individuals including babies born to HIV infected mothers, individuals both on ART and not on ART, and patients with virological failure.

HIV-1 subtype was detected and HIV drug resistance testing was performed with the samples of ART naïve individual, patients with treatment failure of first line anti-retroviral therapy, virologic failure patients and infant born to HIV positive mother. The Viroseq (Abbott diagnostics, Wiesbaden, Germany) and Qiamp (Qiagen, GMBH, Hilden Germany) both the platform was used. Obtained sequence were analysed in the HIV drug resistance database of Stanford University. Clade typing and phylogenetic reconstructions was performed using the REGA sub typing tool of the HIV drug resistance database, and nucleotide sequences were aligned using the Clustal W multiple sequence alignment program. Phylogenetic analysis was conducted using MEGA v3.0 software. The neighbor-joining method and Kimura parameter model were used for tree construction with reliability estimated from 1000 boot strap replicates.

All the obtained sequences were found subtype C with some recombinants. In pre-treatment HIV patients two recombinants of subtype C were submitted to GenBank and accession no (LC570898 & LC529744) was obtained.

The PI major drug resistance mutations detected in the protease gene were M46L and V32I. Whereas accessory PI drug resistance mutations were L23I, L24I and V82Deletion, for the RT gene L74V, M184V D67N, K70N, K70A, K65Q, D67S, L74I,

M184R, M184 Deletion, T215 Deletion, K65Deletion D67DN, L74Q, K65L, M184R, L74Q L74Y were detected as NRTI and E138EA E138R K101KE, K103N, G190GA V179D, Y188L Y181C Y181Deletion, Y188Deletion, G190Deletion Y181L, Y188R, G190G_REQRN Y188C, K238T were detected as NNRT. High variation of major and accessary PI mutations, NRTI- and NNRTI-resistant mutation was observed. NRTI and NNRTI DRMs were met at a frequency of 34 (77.27%) and 15(34.09%) amongst 44 first line ART failure patients, with M184V (34.09%), T215F (25.0%) and K219E (20.45%) being the most frequent among NRTI associated mutations, and Y188L (18.18%), K103N (6.81%) and A98G (6.81%) among NNRTI associated ones. PI DRMs were observed in 5/44 (11.3%) patients, with V82L, V82S and I84V being the commonest.

Virologic failure was detected among 15 samples out of 365 HIV/AIDS patients on second line ART. DR mutations were detected in 5 out of 9 samples and among the rest 4 samples no HIV DR mutation was detected. Among NRTI based drugs, M184V and M41L were predominant mutations (80%). For NNRTI based drugs, A98G and Y181C were predominant (80%) conferring resistance to DLV and NVP. Again for Ps, 154V, A71V, V82A and M46L were seen in 40% of the cases conferring resistance to IDV, SQV, LPV, NFV and ATV. With the predominant subtype C, few recombinant of C, D and J were found in infants born to HIV positive mothers whereas no drug resistance mutations were found in infants.

This study has been conducted with four different kinds of subjects with subtype diversity and implications of ART on their treatment. The pattern of DRM in pre-treatment patients may help to select appropriate ARV drugs at right time. For early identification of virologic failure, mandatory viral load testing is highly recommended by this study. Rapid access of ART in India warrants urgent need of HIV drug resistance surveillance for proper monitoring on adherence to ART that minimize the development of DRMs. HIV diversity may have consequences for diagnosis, pathogenesis, transmission, clinical management, epidemic dynamics and vaccine development.

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Aims and Objectives

Aims:

Studies on Molecular Diversity of Human Immunodeficiency Virus -1

Objectives:

To find out HIV-1 diversity including

(a) Subtype Analysis

(b) Testing of antiretroviral drug resistance mutations

(c) Phylogenetic Analysis

The Study Subjects involved:

(i) ART naïve HIV infected individual.

(ii) HIV infected individuals under ART.

(iii) AIDS patients under ART presenting Virological failure.

(iv) Babies (6 weeks to 18 months) born to HIV positive mothers.

Abbreviation

ADCVI	-	Antibody dependent cell-mediated viral inhibition
ADCC	-	Antibody dependent cellular cytotoxicity
AIDS	-	Acquired Immuno Deficiency Syndrome
APC	-	Antigen presenting cell
ART	-	Antiretroviral therapy
CA	-	Capsid protein
CCR5	-	Chemokine (C-C Motif) Receptor 5
CD	-	Cluster of differentiation
cDNA	-	Complementary DNA
CDC	-	Centre for Disease Control
CD4	-	Cluster of differentiation 4
CD8	-	Cluster of differentiation 8
CDR	-	Complementarity determining region
CRFs	-	Circulating Recombinant Forms
CTL	-	Cytotoxic T lymphocytes
CXCR4	-	CXC Chemokine Receptor 4
C1-C5	-	Conserved regions 1 to 5
DEAE	-	Diethylaminoethyl
dH_2O	-	Distilled water
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribose nucleic acid
dsDNA	-	Double stranded deoxyribose nucleic acid
dNTP	-	Deoxy nucleotide
EDTA	-	Ethylene Diamine Tetra acetic Acid
ELISA	-	Enzyme linked immunosorbent Assay
Env	-	Envelope

FACS	-	Fluroscense assisted cell sorter
FITC	-	Fluroscein iso thiocyanate
FCS	-	Fetal calf serum
gp 120	-	Surface glycoprotein 120
gp 160	-	Envelope glycoprotein 160
gp 41	-	Transmembrane glycoprotein 41
GRID	-	Gay-Related Immune Deficiency
HAART	-	Highly Active Antiretroviral Therapy
HIV	-	Human Immunodeficiency Virus
Hr	-	Hours
HRP	-	Horse radish peroxidase
HTLV	-	Human T-Cell Leukaemia Virus
IAVI	-	International AIDS Vaccine Initiative
IN	-	Integrase
LB	-	Luria Bertani
LTNP	-	Long term non progressors
LTR	-	Long terminal repeat
MA	-	Matrix Protein
MHC	-	Major Histocompatibility Complex
MPER	-	Membrane Proximal External Region
NACO	-	National AIDS Control Organization
NC	-	Nucleocapsid Protein
Nef	-	Negative factor
NIH	-	National Institute of Health
NK	-	Natural killer cell
NNRI	-	Non-Nucleotide reverse Transcriptase inhibitor
NRTI	-	Nucleotide reverse Transcriptase inhibitor

PBS	-	Phosphate Buffered Saline
PFA	-	Paraformaldehyde
PLHIV	-	People living with HIV
Pol	-	Polymerase
PR	-	Protease
PI	-	Protease Inhibitor
R5	-	CCR5 tropic
RNA	-	Ribonucleic Acid
RT	-	Reverse transcriptase
rpm	-	Revolution per minute
RPMI	-	Roswell Park Memorial Institute
SIV	-	Simian Immunodeficiency Virus
SP	-	Specificity protein
TAE Buffer	-	Tris-Acetate-EDTA Buffer
Tat	-	Trans-activator of transcription
TAR	-	Trans-activating response element
TCID50	-	50% Tissue Culture Infectious Dose
USA	-	Unites States of America
V1 – V5	-	Variable regions 1 to 5
WHO	-	World Health Organization
X4	-	CXCR4 tropic
kDa	-	KiloDaltons
µl	-	Micro liter
µM	-	Micro meter
ng	-	Nanogram
nm	-	Nanometer
nM	-	Nanomole

Introduction

Introduction

Human Immunodeficiency Virus (HIV) is a retrovirus that targets and alters the immune system, increasing the risk and impact of other infections and diseases. Without treatment, the infection might progress to an advanced disease stage called Acquired Immunodeficiency Syndrome (AIDS).

In recent years, all other form of immunodeficiency has been over shadowed by an epidemic of severe immunodeficiency caused by infectious agent called HIV. As HIV is a retrovirus, it carries their genetic information in the form of RNA. When the virus enter into a cell, the RNA is reverse transcribe to DNA which integrated into the cell genome and is replicated along with the cell DNA. Person with HIV showed a marked deficiency in cellular immune responses and a significant decrease in the subpopulation of T-cells that carry the CD4 marker (T helper cells). Progression of HIV infection to AIDS has revealed a dynamic interplay between the virus and the immune system. The initial infection causes dissemination of virus to lymphoid organs and a resultant strong immune response which involves both antibody and cytotoxic CD8+ T lymphocytes to keep the viral infection in check. Despite high rate of replication, the virus is kept in check by immune system throughout the chronic phase of infection. Low level of virus correlates with a longer time in which the infected individual remains free for opportunistic infection. The virus eventually breakthrough the host immune defense, resulting in an increase in viral load, a decrease in CD4+ T cell numbers, increase opportunistic infection, and death of the patient.

Origin and Discovery:

HIV originally came from a virus, Simian immunodeficiency virus (SIV) causes immunodeficiency disease in particular chimpanzees in West Africa and originally transmitted to humans through the transfer of blood through hunting [Worobey M.et al.

2010]. Over the decades, the virus spread through Africa, and to other parts of the world. Research found that HIV is related to SIV and there are many similarities between the two viruses. HIV-1 is closely related to a strain of SIV found in chimpanzees, and HIV-2 is closely related to a strain of SIV found in sooty mangabeys [Sharp, P.M. et. al 2011].

However, it wasn't until the early 1980s, when rare types of pneumonia, cancer, and other illnesses were being reported to doctors that the world became aware of HIV and AIDS. This timeline highlights some of the major events and discoveries in HIV and AIDS since this time. In 1981 US, reporting of unusually high rates of the rare forms of pneumonia and cancer in young gay men begins. The disease is initially called Gay-Related Immune Deficiency (GRID) because it is thought it only affects gay men. Cases are also reported in Injection Drug Users by the end of the year. In 1982, this disease is renamed as Acquired Immune Deficiency Syndrome (AIDS). It is realized that the infection can be sexually transmitted and caused by HIV. Cases are reported in blood transfusion recipients. In 1983 it is discovered that women can become infected with AIDS through heterosexual sex. 1985 the first International Conference on AIDS is held in Georgia, USA. In1986 it is discovered that HIV can be passed from mother to child through breast-feeding. In 1988 the first World AIDS Day is held on December 1st. In 1991 the red ribbon became the international symbol of AIDS awareness, intended to be a symbol of compassion for people living with HIV and their careers.

Current Epidemic Status: Global and National:

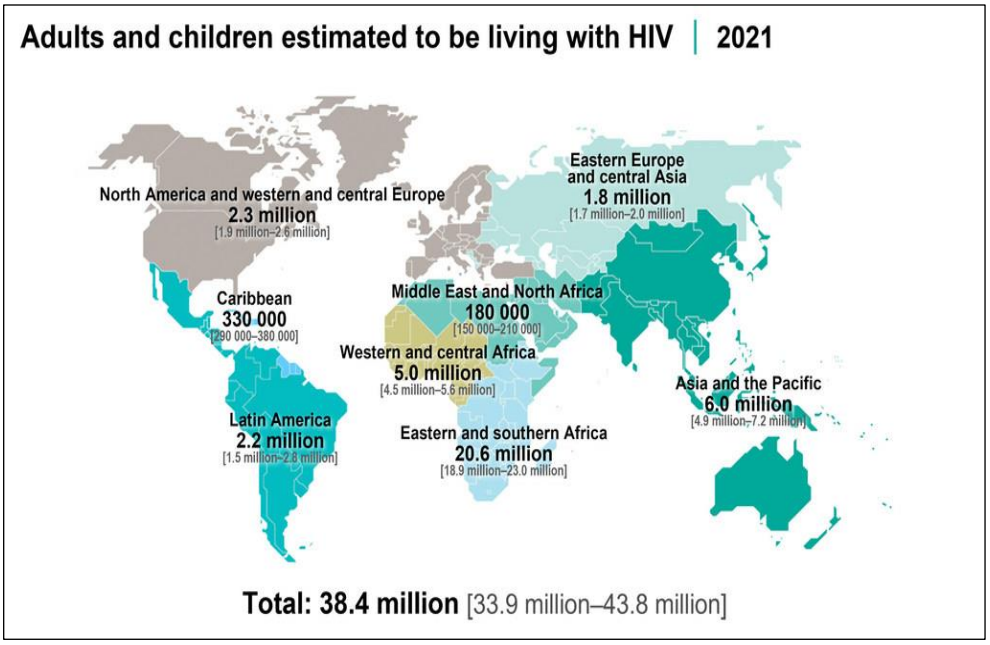

Figure 1: HIV Estimation Globally [UNAIDS 2021]

Today, there are approximately 38.4 million (33.9 million – 43.8 million) people currently living with HIV, and tens of millions of people have died of AIDS-related causes since the beginning of the epidemic [UNAIDS Global HIV & AIDS statistics-Fact Sheet, 2021]. Over the past two decades, in particular, major global efforts have been mounted to address the epidemic, and significant progress has been made. The number of people newly infected with HIV, especially children, and the numbers of AIDS-related deaths have declined over the years, and the number of people with HIV accessing antiretroviral therapy is 28.7 million which is increased to 7.8 million in 2010. While there have been significant declines in new infections since the peak in 1996, there are still about 1.5 million new infections in 2021. Recent data shows that the pace of decline in new infections is too slow to reach global targets. Furthermore, the pace of decline varies by age group, sex, and region. Young people, ages 15-24, account for approximately a third of new HIV infections, and in some areas, young women are disproportionally impacted. In sub-Saharan Africa, young women 15+ years account for a quarter of all new HIV infections in the region in 2021, even though they

represent only 10% of the population. Globally, there were 2,730,000 children are living with HIV, Number of new infections among children is 310,000 and 110,000 AIDS-related deaths in 2021. Since 2010, new HIV infections among children have declined by 52% [Fact Sheet 2021, UNIDS].

Despite being home to the world's third-largest population suffering from HIV/AIDS (with South Africa and Nigeria having more), the AIDS prevalence rate in India is lower than in many other countries. India's large population has led to a large number of affected people while the overall Prevalence rate is low. In 2014, The spread of HIV in India is primarily restricted to the southern and north-eastern regions of the country and India has also been praised for its extensive anti-AIDS campaign [Clinton lauds India Aids campaign". 26 May 2005].The US$2.5 billion National AIDS Control Plan III was set up by India in 2007 and received support from UNAIDS [Driving forward an effective AIDS response, 2018].

The main factors which have contributed to India's large HIV-infected population are extensive labor migration and low literacy levels in certain rural areas resulting in lack of awareness and gender disparity.[Source of Infections in AIDS cases in India, Embassy of India] The Government of India has also raised concerns about the role of intravenous drug use and prostitution in spreading AIDS, especially in north-east India and certain urban pockets.[8] The HIV/AIDS epidemic in India began in 1986-1987 with the detection of the first HIV infection in Chennai and the first AIDS Case in Mumbai. Since then the HIV epidemic has spread to rural and urban areas, infecting high-risk groups as well as the general population. However, nearly 25 years since the epidemic appeared in India, the disease has not reached the proportions predicted by experts across the world. The Indian epidemic is still a concentrated epidemic with high HIV prevalence remaining in the high-risk group. In 2021, the estimated adult prevalence, in the general population, is 0.21%t and the total number of people living with HIV/AIDS (PLHIV) is estimated 24 lakh. India is estimated to have

around 62.97 thousand annual new HIV infections among adults and PMTCT need are estimated 20,612 in 2021. The adult HIV prevalence in India is declining from estimated level of 0.32% (0.26 – 0.40) in 2010 through 0.21% (0.17-0.25) in 2021. Adult HIV prevalence at a national level has declined notably in many states, but variations still exist across the states. As per the recently released, India HIV Estimation 2021 report, adult (15–49 years) HIV prevalence in India is estimated at 0.21% (0.17 – 0.25) in 2021. The adult HIV prevalence is estimated at 0.22% (0.18-0.28) among males and at 0.19% (0.15-0.23) among Females. The adult HIV prevalence at national level has continued its steady decline from 2010 to 2021 [India HIV Estimates 2021, Fact sheet].

HIV Classification

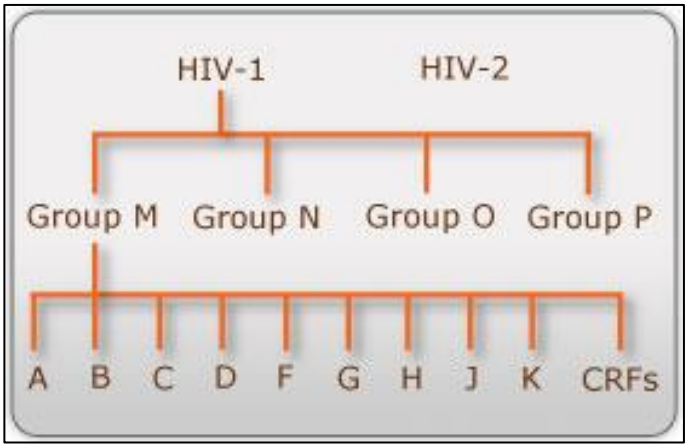

Figure 2: Classification of HIV

There are two types of HIV; HIV-1 andHIV-2. HIV-1 is further divided into three main groups; major (M), outliers (O) and new groups (N). An additional fourth group, 'P,' has been documented in a Cameroonian woman living in Paris. Group M is most common type of HIV in the world, whereas groups N and O are mainly confined to West and Central Africa and form very small minorities. Group M is further classified into subtypes A-K based on phylogenetic relatedness. The image is adopted from Kerina Duri. et.al.2013

Immunopathogenesis

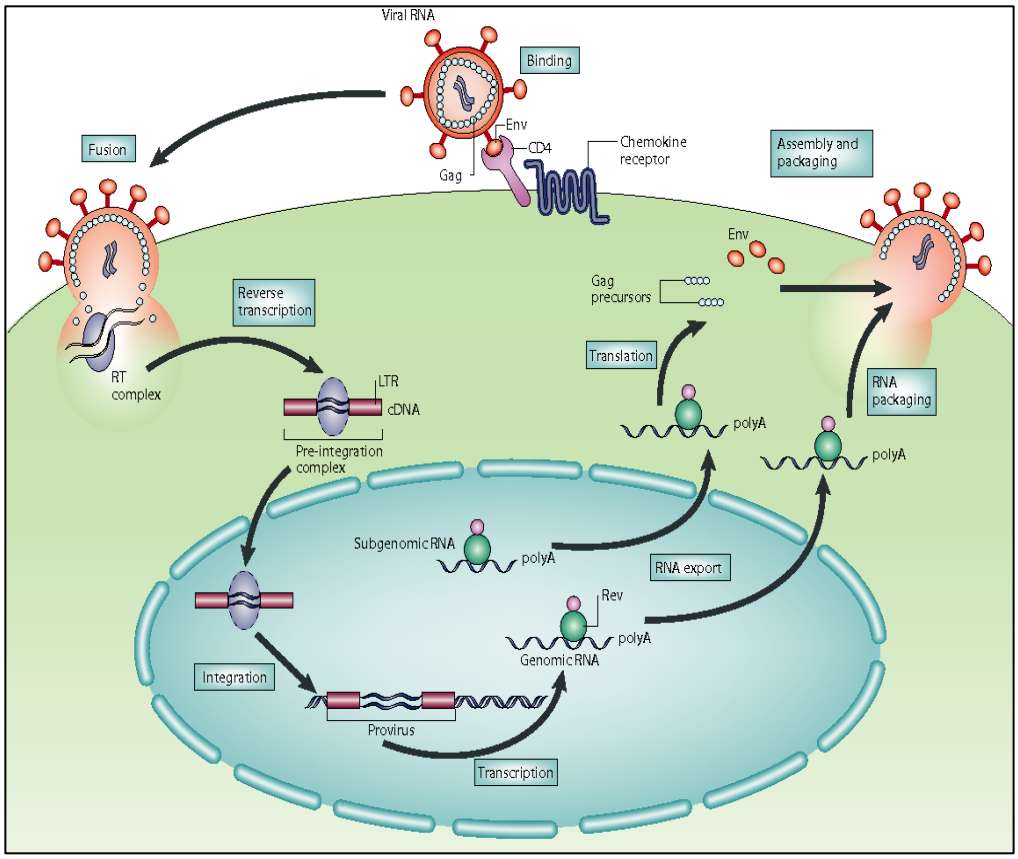

Figure 3: Schematic illustration of HIV lifecycle within an infected cell, RT- Reverse Transcriptase; LT- Long terminal repeat. (The image is adopted from Nilsson, Jakob. "On the immunopathogenesis of HIV infection." (2006).

Disease Progression and Immune Cell Destruction

Binding and Fusion: The first step in fusion involves the high-affinity attachment of viral gp120 to a CD4 molecule. This leads to conformational changes that expose the co-receptor (CCR5/CXCR4) binding sites present on the surface of a CD4 T lymphocyte. This leads to the fusion of the virus envelope with host cell membranes and releases the viral genetic material (RNA) in the protoplasm of the host cell.

Reverse Transcription: In the protoplasm, the viral reverse transcriptase enzyme transcribes the single-stranded HIV RNA into a double-stranded c-DNA that subsequently moves to the nucleus.

Integration: The HIV enzyme "integrase" integrates the HIV DNA with the host cell's DNA. The integrated HIV DNA is called provirus. The provirus may remain inactive for several years, producing few or no new copies of HIV.

Transcription: When the host cell is activated, the provirus integrates with the host genome and transcribes with the host's RNA polymerase to create copies of HIV RNA, as well as HIV mRNAs. These are then exported from the nucleus to protoplasm.

Protein Synthesis and Assembly: mRNAs code for viral polyproteins in the protoplasm. An HIV enzyme "protease" cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.

Budding: The final step of this viral cycle is assembly of new HIV -1 virions and bud formation from the host cell. The budded virions are needed to be cleaved into the actual matrix, capsid and nucleocapsid proteins. This cleavage is mediated by the packaged viral protease and can be inhibited by antiretroviral drugs of the protease inhibitor. (Saha M.et.al.2019)

Transmission

HIV is transmitted in human body fluids by three major routes:

- Unprotected Sex: Sexual intercourse through vaginal, rectal, or penile tissues;
- Sharing injecting equipment: Direct injection with HIV-contaminated drugs, needles, syringes, blood or blood products;

- Passed from mother-to-baby during pregnancy: childbirth and breastfeeding: from HIV-infected mother to fetus in uterus, through intrapartum inoculation from mother to infant or during breast-feeding (naco.gov.in)

HIV Symptoms

The symptoms of HIV vary, depending on the individuals and what stage of the disease is in the early stage, the clinical latency stage, or AIDS.

Early stage: - About 40% to 90% of people have flu-like symptoms within 2-4 weeks after HIV infection. Other people do not feel sick at all during this stage, which is also known as acute HIV infection. Flu like symptoms can include; fever, chills, rashes, night sweats, muscle aches, sore throat, fatigue and swollen lymph nodes.

Clinical latency stage: - after the early stage of HIV infection, the disease moves into a stage called the clinical latency stage (also called "chronic HIV infection"). During this stage, HIV is still active but reproduces at very low levels people with chronic HIV infection may not have any HIV related symptoms, or only mild ones.

Progression of AIDS: - Recurring fever or profuse night sweats, Extreme and unexplained tiredness with weight loss, Prolonged swelling of the lymph glands in the armpits, groin or neck, Diarrhea that lasts for more than a week, Sores of the mouth, anus, or genitals, Pneumonia, Red, brown, pink blotches on or under the skin, mouth, nose or eyelids (Saha M.et.al.2019)

Detection of HIV

The detection procedure can be classified as screening assays, which are designed to detect all individuals and the confirmatory assays which are manufactured to

differentiate between the individual of false reactive in screening assays from those who are truly infected [Niel. T. et.al. 2005]

Detection of HIV is based on assays for presence of HIV antibodies. Currently, standard of these diagnostic assays conforms national and international standards. [Maity S. et.al.2011, Banerjee S. et.al.2022, Bhatta M. et.al.2021].

In this thesis, the detection of HIV antibodies is primary work done in Chapter 3 and chapter 4. Third generation kits for detection of HIV has been selected followed by comparing the sensitivity and specificity described in published article [Nandi S. et.al.2014]

Prevention and Treatment

- Educate yourself and others.
- Know the HIV status of your sexual partner.
- Consider male circumcision.
- Use a clean needle.
- Be cautious about blood products.
- Get regular screening tests.
- Use condom and avoid unprotected sex
- Adherence to ART

Treatment: The major treatment for HIV infected patient is Anti-Retroviral Treatment (ART). The major classes of drug available in the NACO are: -

- Nucleoside reverse transcriptase inhibitor (NRTI) acts as a DNA chain terminator. They are faulty versions of building blocks that HIV needs to make more copies of it such as Zidovudine (Retrovir).

- Non-nucleoside reverse transcriptase inhibitor (NNRTI) inhibits the HIV reverse transcriptase enzyme by binding to it such as Nevirapine (Veramonte).
- Protease inhibitor (PI) binds to the active site of the protease and prevents maturation like Lotinavir (Kaletra).
- Newer classes of anti-HIV drugs include fusion inhibitors to stop the integration of virus DNA.

The National AIDS Control Organization (NACO) initiated free antiretroviral treatment in April 2004.it has established 425 ART centers all over India.

ART Goals

Clinical goals:-Increased survival and improvement in quality of life.

Virological goals: -Greatest possible sustained reduction in viral load.

Immunological goals:-Immune reconstitution, that is both quantitative and qualitative.

Therapeutic goals: -Rational sequencing of drugs in a manner that achieves clinical, virological and immunological goals while maintaining future treatment options, limiting drug toxicity and facilitating adherence.

Preventive goals: -Reduction of HIV transmission by suppression of viral load. (National guideline for HIV care and treatment 2021).

Biosafety Practices in the Laboratory

Biosafety Practices in the laboratory

Safety in the laboratory requires every employee's participation and cooperation. Non-compliance with safety precautions not only endangers the individual, but also compromises the health and safety of fellow workers. All staff of the laboratory shall follow the following:

- Drinking or eating in the laboratory are not allowed. A sign board to this effect should be pasted at prominent place at all laboratory areas.
- Personal Protective Equipment (PPE)-gown, gloves, goggles, face masks and shields should be available in the laboratory and should be accessible to the worker. Always use protective equipment that is provided for working with hazardous materials. Wear full sleeved laboratory coats buttoned up and gloves while working in the laboratory.
- Wipe the working area with disinfectants (70% alcohol / 0.1% sodium hypochlorite solution) at the beginning and end of the laboratory session and record it.
- Avoid any activity that introduces objects into the mouth, e. g., mouth pipetting.
- Cover any open cuts on hands and other exposed skin surfaces with a water-resistant dressing.
- Carry out all procedures in a way so as to minimize the risk of spills, splashes and the production of aerosols. In case of spills, manage the area with 1% sodium hypochlorite solution.
- Always wash your hands before leaving the laboratory.
- Benches should be clear of all non-essential materials including books and notes.
- Be familiar with the location and operation of eye washers, safety showers and first aid box.
- All laboratory employees should be vaccinated for Hepatitis B and Tetanus vaccine.
- A first aid box should be kept with adequate materials and medicines with the expiry date.
- Safe handling of specimens in the laboratory (suggestive points to be included given) Open the specimen bottles only at working areas, identify each specimen, avoid spillage, breakages, clutter fire work area etc.
- Biomedical wastes generated in the HIV laboratory should be autoclaved and disposed by maintaining State pollution Control board guidelines.
- Entry of laboratory should be restricted by authorized persons only.
- In case of an accidental injuries with infectious material, report immediately to the designated Medical Officer (Biosafety Manual, WHO, Geneva 4th Edition, 2020).

Chapter 1

Literature review on Molecular Diversity of HIV-1

Review of Literature

Human Immunodeficiency Virus differs from many viruses because of high genetic variability. This diversity is a result of its fast replication cycle, with the generation of about 10^{10} virions every day, coupled with a high mutation rate of approximately 3 x 10^{-5} per nucleotide base per cycle of replication and recombinogenic properties of reverse transcriptase [Perelson AS *et al.*, 2008]. High affinity attachment of the CD4 binding domains of gp120 to CD4 is the first step in fusion. The glycoprotein 120 binds to integrin $\alpha_4\beta_7$ activating LFA-1, the central integrin involved in the formation of virological synapses and this synapses enable efficient cell to cell spreading of HIV-1. [Arthos J. *et al.*, 2008].

For HIV treatment as well as prevention, efficiency of antiretroviral therapy (ART) is outstanding and thus setting of targets including 90% PLHIV should know their serostatus, among them 90% should receive continuous ART and 90% among them should have viral suppression are remarkable (UNAIDS, 2016-2021 Strategy). But the overall ART response is hampered by HIV drug resistance [WHO, HIV drug resistance report 2017].

HIV Structure

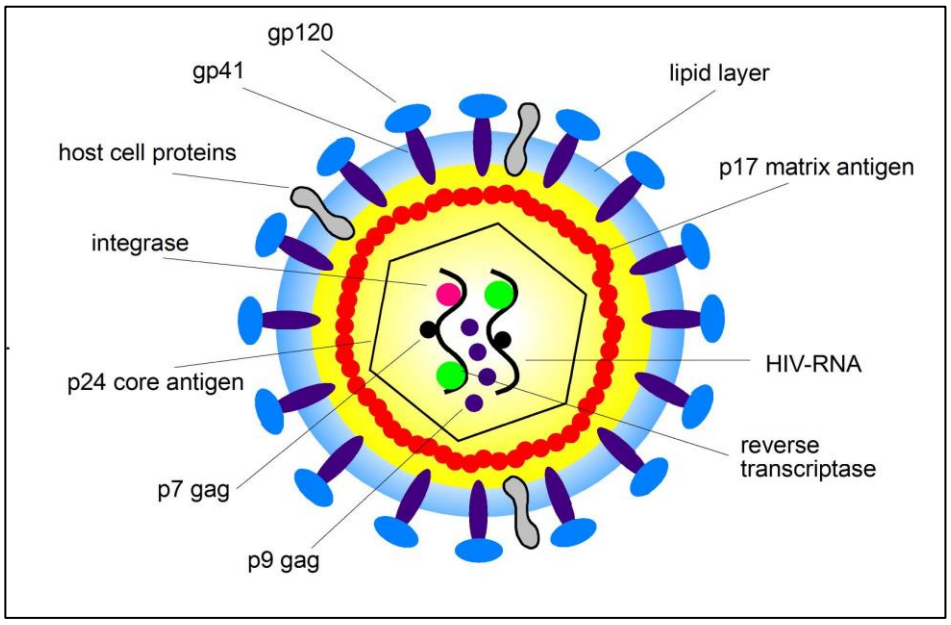

Figure 1.1: Structure of HIV [Christian, Parfum. (2019). Community-Based HIV Care.]

Human Immunodeficiency Virus is a genus of lentivirus which is a part of Retroviridae [International Committee of Taxonomy of Viruses, 2002]. Many species are infected by lentiviruses, which are characteristically responsible for long-duration illnesses with a long iincubation period that causes the Aquired Immunodeficiency Syndrome (AIDS),a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive [Weiss RA et.al.1993 & Douek DC et.al.2009]. The structure of HIV is different than other retroviruses although it is transmitted as enclosed in a lipid bilayer envelope derived from host cell membrane. The diameter of the virus is around 120 nm and the shape is roughly spherical with idiosyncratic cone shaped. [Brian G Turner.et.al.1999]

HIV is an enveloped virus. The virus envelope is composed of two phospholipid layers derived from the host cell membrane. The envelope also contains the trimers of an envelope coated protein, glycoprotein (gp) 160. Gp160 is composed of two subunits, gp120 and gp41. While gp120 has external protein and contains sites that bind CD4 cells and co-receptors on the surface of human CD4 T cells, gp41 is membrane bound protein. Inside the viral envelope there is a layer called the matrix, which is made from the protein p17. The viral core (or capsid) is usually bullet-shaped and is made up of protein p24. Inside the core are three enzymes required for HIV replication: Reverse Transcriptase (RT), integrase and protease. Also held within the core is HIV genetic material which consists of two positive strands of single stranded Ribonucleic Acid (RNA) [Fanales-Belasio.E, 2010 HIV virology and pathogenetic mechanisms of infection: a brief overview].

The production of HIV in cellular level occurs by grasping the cell surface receptor which is followed by various reactions till the new virus particle comes out to make a mature virus [EngelmanA.et.al.2012].

HIV Life Cycle

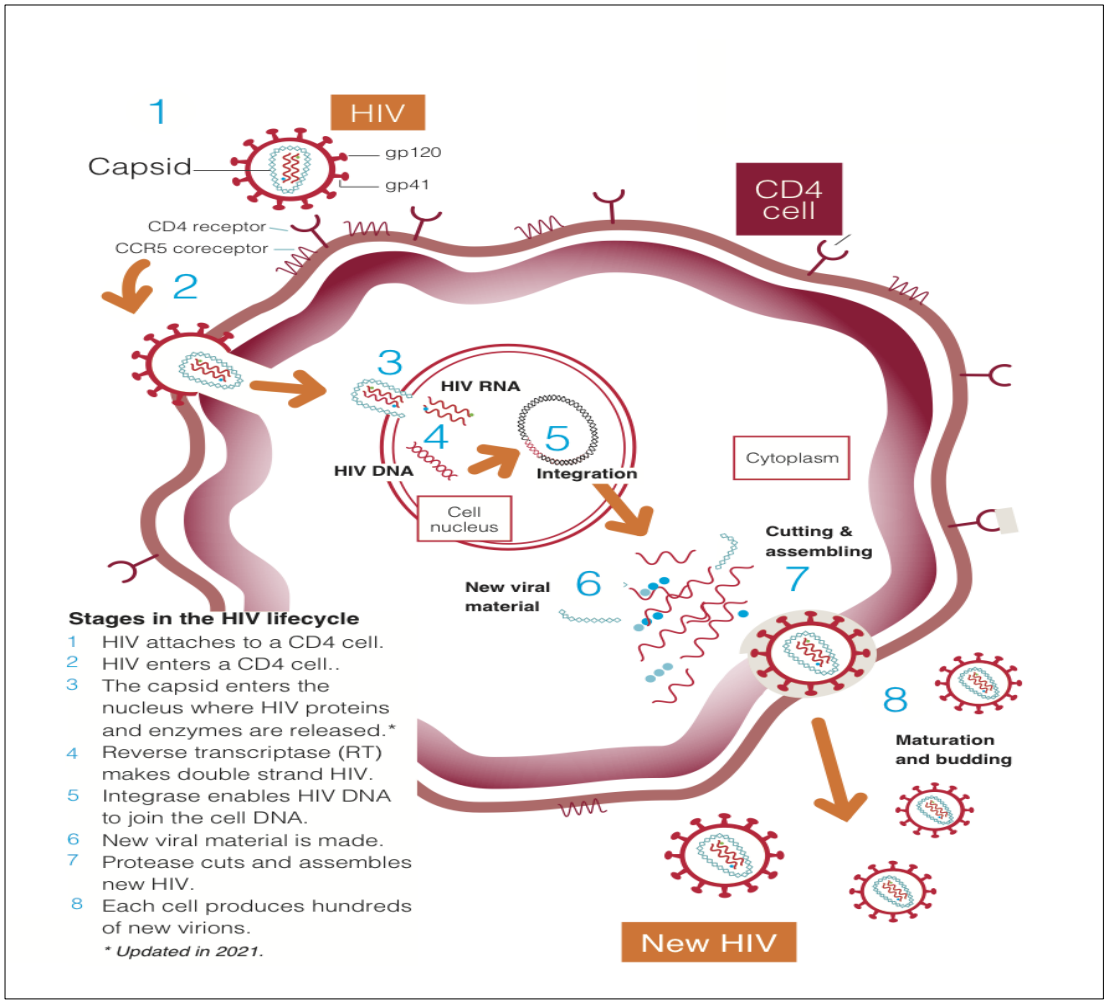

Figure 1.2: Key steps in HIV Life Cycle (Source of image is https://i-base.info/)

In the first step, HIV attaches and enters into a CD4 cell. Then capsid enters the nucleus where HIV proteins and enzymes are released. In the next step, Reverse Transcriptase (RT) makes double stranded HIV. In this step, Integrase helps HIV DNA to join the cell DNA. Then Protease cuts and accumulates new HIV. Now each cell produces more than hundreds of new virions [https://i-base.info/].

HIV genome organization

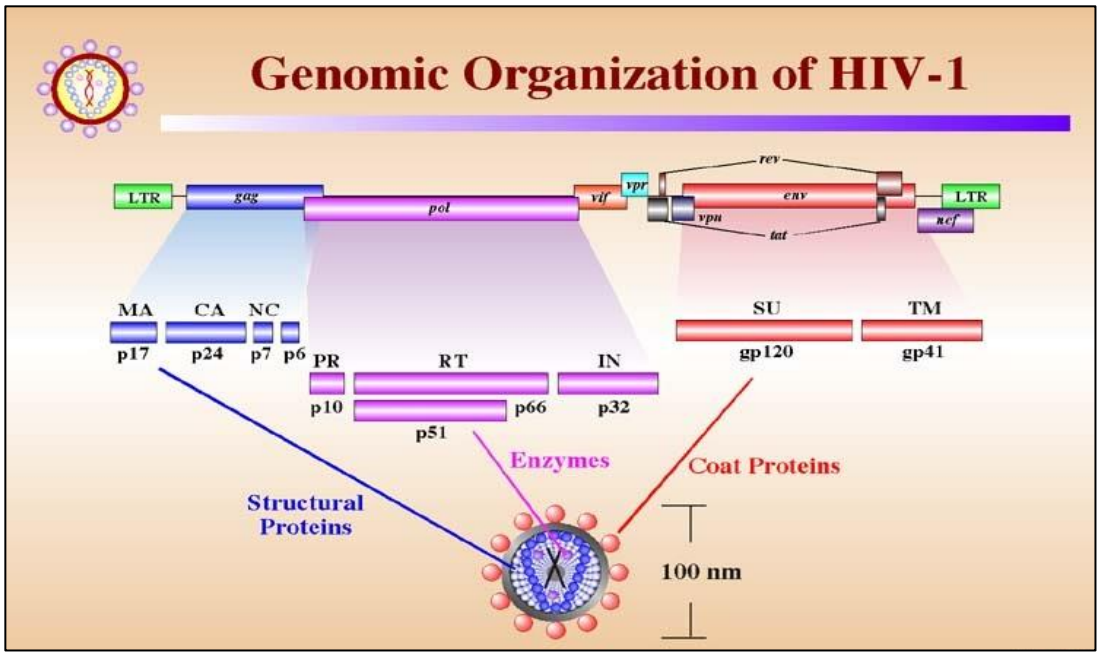

Figure 1.3: Schematic representation of HIV genome (Image is adopted from web.stanford.edu.)

HIV genome is 9.2 kb in size and consists of 9 genes. These 9 genes finally produce 15 proteins: *env*- envelope; *gag*- group specific antigen; *LTR*- Long Terminal repeats; *nef*- negative factor; pol- polymerase; rev- regulator of expression of viral proteins; *tat*- transactivator of transcription; *vif* – virus infectivity factor, *vpr* – viral proteins R; *vpu* – viral proteins U.

These 15 proteins divided into sub categories: (i) structural proteins (Gag, Env, Pol), (ii) Regulatory Proteins (Tat, Rev) and (iii) Accessory proteins (Nef, Vif, Vpr, Vpu).

Envelope (Env): It is 2570 nucleotides in size. The envelope precursor is known as gp160 and is processed by a cellular protease that results in production of two proteins; (a) surface envelope protein which is known as gp120 and (b) transmembrane glycoprotein called gp41. The gp 120 contains determinants that interact with T cell receptors and gp41 interacts with

co-receptors. The gp120 primarily binds with the target cell due to co-receptor interaction that includes conformational changes in gp41. These changes promote formation of a gp120/ gp41 glycoprotein complex that leads to fusion of the virus to the host cell membrane [Animal Biotechnology: Models in Discovery and Translation, 2013]

Group pecific Antigen (gag): The gag gene encodes for Pr55Gag, which is a polyprotein precursor. Pr55Gag is cleaved, leading to formation of four proteins: p6, p7, p17 and p24 as well as two spacer peptides, p1 and p2. P17 is a matrix protein, p24 is a capsid protein and p7 is a nucleocapsid and p6 is known as Vpr binding protein. The N terminal of matrix protein is mainly responsible for targeting and binding to plasma membrane [Ganser-Pornillos et.al. 2008].

Polymerase (Pol): The genomic location of polymerase is from 1839-4642 nucleotides. The individual pol encoded enzymes, viral proteases (PR or p10), reverse transcriptase (RT or p64) and integrase (IN or p32) are cleaved from the polyprotein precursor by viral proteases. Integrase protein promotes insertion of linear but double stranded pro-viral DNA into the chromosome of the host cell. Integration is absolutely necessary step for viral replication because integrase mutant viruses fail to spread infections [Dulude at.al. 2008]

Negative Factor (Nef): Nef is an accessary proteins in HIV are expressed during the early stages of replication in host cells. Nef down regulates expression of cell receptors like CD4, CD8, CCR5, CXCR4 etc. The functions of Nef protein are as follows: It perturbs endocytocis, it modulates signal transduction pathways in infected cells, it enhances viral infectivity and it supports fusion of HIV -1 to target cells [Waheed and Freed, 2009].

Regulator of Expression of Viral Proteins (Rev): Rev is encoded by two exons, both of which are essential to producing functional proteins. Rev is a regulatory protein and is essential for regulation of viral replication. It contains two functional domains: a) arginine rich domain

that binds with viral RNA and supports nuclear localization and b) a leucine rich domain that is hydrophobic and meditates nuclear export of viral RNA [Schwartz et. al., 1990].

Transactivator of transcription (Tat): Tat is a regulatory protein essential for regulation of viral replication. It comprises 259 nucleotide sequences. Tat protein consists of several functional domains. Secreted Tat may be taken up by neighboring cells, therefore Tat effects both infected and non- infected cells [Levy, 2007].

Viral infectivity Factor (Vif): Vif is an accessary protein. It is comprised of 578 nuceotides. Vif has a major role in the production of infection-competent new virions from infected cells. Vif is expressed during late stages of HIV replication, and is localized in the cytoplasm of infected cells. Vif appears to act during viral assembly in virus-producing cells, or subsequently in virion maturation to produce virions competent for reverse transcription in the target cells [Rose et.al.2004].

Viral Protein U (Vpu): Viral protein U is a type-1 integral membrane phosphoprotein unique to HIV. It enhances pathogenesis in *vivo,* even though it is not an essential protein. The Vpu protein usually gets expressed during replication in the host cell. It performs two major functions during HIV replication: a) It enhances the release of viral particles, and b) It promotes CD4 degradation [Votteler and Schubert, 2008].

Viral Protein R (Vpr): Vpr is an accessary protein of HIV and is not essential for viral replication. Vpr implied in various biological functions like transcription of new viral genomes, apoptosis induction, disruption of cell cycle control, induction of defects in mitosis, nuclear transport of the pre – integration complex (PIC), facilitation of reverse transcription, suppression of immune activation, as well as reduction of HIV mutation rate [Le Rouzic an Benichou, 2005].

Long Terminal Repeats (LTR): *LTR* is present on either side of viral genome. It harbors *cis*-acting elements, which are required for RNA synthesis, and is the initiation site for transcription for the viral genome. LTR consists of three regions: U3 (Unique, 3' end), R (repeated), and U5 (Unique, 5' end). Various elements present in U3 help in direct binding of RNA polymers II (Pol II) to DNA templates [Marcello et. al. 2004].

Prevalence of HIV

In 2021, as per estimation by UNAIDS, globally 38.4 million people were living with HIV in 2021, among them the newly infection was 1.5 million, 560,000 were died from AIDS related illness [UNAIDS epidemiological estimates 2022].

According to the data published by National AIDS Control Organization, Ministry of Health and Family Welfare, Govt. of India, the estimated HIV prevalence (15-49 years) is 0.21% antenatal clinic (ANC) attendees which considered a proxy for prevalence among the general population in the country whereas in the northeast states of Tripura, Meghalaya, Arunachal Pradesh, Assam, Sikkim, Mizoram and the Union Terriitory of Dadra and Nagar Haveli, Daman and Diu an increasing trend is projected and in 2021, AIDS related death are estimated at 41.97 thousand [India HIV Estimates 2021: Fact Sheet. New Delhi: NACO].

HIV Diversity

Origin of HIV types, Groups and Subtypes

HIV-1 is distinguished from HIV-2 because it carries the auxiliary gene vpu, whereas HIV-2 lacks this gene but carries vpx [Levy JA *et al.*, 2007]. The other genes have less than 25% sequence similarity when homologues are compared between the two groups. HIV-1 came to humans from great apes in central Africa, whereas HIV-2 came from sooty mangabey monkeys in West Africa and also gave rise to simian immunodeficiency virus in Asian

macaques (SIVmac) via common housing in captivity in primate centers in USA. HIV-1 has independently crossed into humans at least four times (HIV-1 groups M, N, O and P) from chimpanzees or for P possibly from a gorilla [Sharp PM *et al.*, 2010] and HIV-2 at least eight times.

In another study three groups of HIV-1 have been identified on the basis of differences in the envelope (*env*) region: M, N, and O. Group M is the most prevalent and is subdivided into eight subtypes, based on the whole genome, which are geographically distinct. The most prevalent are subtypes B (found mainly in North America and Europe), A and D (found mainly in Africa), and C (found mainly in Africa and Asia); these subtypes form branches in the phylogenetic tree representing the lineage of the M group of HIV-1. Confection with distinct subtypes gives rise to circulating recombinant forms (CRFs). [Thomson MM *et al.*, 2002]

The geographic origin of HIV-1, group M, to chimpanzees in southeast Cameroon. HIV-1 group M has given rise to the AIDS pandemic that has infected around 60 million individuals to date. In contrast, HIV-1 group O has infected around 10 000 individuals, and HIV-1 groups N and P far fewer. It is estimated that the most recent common ancestor of HIV-1 group M started to replicate approximately 100 years ago as a founder virus in a single individual [Korber B *et al*., 2001].

The virus found fertile social conditions in the former Belgian Congo to spread both parenterally via injections and sexually [Pepin J. 2011]. It gave rise to the HIV-1 group M subtypes (also called clades) A–K, some of which are recombinant viruses (E, I and possibly G) [Korber B et al., 2001]. All are present in the Congo. Generally, subtypes or clades are defined as HIV strains that show greater than 30% genome diversity [Robertson DL. *et. al.*, 2000].

HIV type-1 (HIV-1) shows a greater degree of genetic and antigenic diversity which reflects a high mutation rate during viral replication with a large turnover of virus, and a high tolerance of variation while maintaining reproductive capacity [Ndung'u T *et. al.* 2012].

More transmissible HIV-1 subtypes

The emergence of new variants reflects HIV-1 prevalence, subtype epidemiology and risk-behaviour patterns is different in respect of geographical areas which indicates that certain subtypes may have a transmission advantage while others display higher replicative efficiency [Geretti *et al.,* 2006,].

Certain subtypes may be more transmissible than others, particularly subtype C. The first AIDS cases recognized in South Africa resulted from infection by subtype B imported from the west and mainly infected gay men in the white community. In contrast, the major epidemic of subtype C so prevalent today in the general population only began to spread through South Africa during the 1990s. In fact, subtype C has become the most prevalent virus across eastern and southern Africa from the Horn to the Cape and now represents over 50% of all HIV-1 infections globally [Hemelaar J *et al.* 2011].

HIV-1 subtypes: difference in Virulence

All the subtypes and CRFs of HIV-1 group M can eventually cause AIDS in the majority of untreated infected persons. However, there is evidence of slight differences in the virulence or the rate of progression to AIDS between subtypes. For instance, both subtype A and D occur in the same host populations in Uganda, but people with subtype D tend to develop AIDS earlier [Kaleebu P, *et. al.* 2001].

Subtype D infection also progresses more rapidly in London in the UK [Easterbrook PJ et. al. 2010], where, largely owing to immigration, the degree of HIV-1 diversity is equivalent to that in central Africa [Dreja H *et. al* 2010].

There is also suggestive evidence that the virulence of HIV-1 has increased during the 30-year course of the AIDS pandemic. A recent meta-analysis indicates that modern day infections tend to cause a more rapid rate of CD4 T-lymphocyte decline and increased viral load [Herbeck JT *et al.* 2012].

Subtype distribution in Indian Scenario

Genetic analyses of HIV-1 sequences circulating in India have been limited. Initial reports indicated that viruses from India were more closely related to those identified in South Africa than to those in North America or central Africa. The study conducted at Calcutta, Eastern India on HIV-1 env sequences V3-V4 region of viral envelope revealed that the identification of conserved signature amino acid positions could assist epidemiologic tracking and has implications for the development of a vaccine against subtype C HIV-1 in India. [Shankarappa R. *et al.* 2001,].

The studies on Prevalence of HIV Drug Resistance Mutations in HIV Type 1 Isolates in Antiretroviral Therapy Na¨ıve Population from Northern India and showed 97% of HIV-1 strain belongs to subtype C but very few studies are reported from our country where predominant is the HIV 1 subtype C [Sinha S.*et. al*. 2012].

The genotypic variation of circulating Indian subtype C viruses revealed by a study conducted in Southern India. This study determined the prevalence of mutations at protease and reverse transcriptase drug resistance positions in antiretroviral drug-naive individuals in

southern India by amplification of the *pol* region of the genome from plasma HIV-1 RNA in 50 patients. All sequences clustered with HIV-1 subtype C [Balakrishnan. P. et.al., 2005].

The data published on Genomic diversity of human immunodeficiency virus type-1 in India revealed that out of the 125 samples, 98 (78.4%) were HIV-1 subtype C, 11 (8.8%) were subtype B, 3 (2.4%) were subtype A and 2 (1.6%) were subtype E.[Sahni AK. *et al.*, 2002].

The study conducted in Maharashtra, India revealed that the newly transmitted Indian Envs are antigenically complex which formed a phylogenetically distinct genetic lineage within subtype C [Kulkarni S. S., *et al*. 2009].

The presence of variant subtypes in Indian patients sheds light on the transmission routes of HIV-1 to India. The predominant strain of HIV-1 in India belongs to subtype C and little interpatient nucleotide sequence divergence in the majority of cases suggests recent spread of HIV-1 in Punjab, India. This study emphasizes on the first evidence for non-C subtypes in the Indian population with two epidemiologically linked samples remaining unclassified for any existing env subtype [Jameel, S.et.al. 1995]

In Africa, the regional distributions of individual subtypes and recombinants are broadly stable, although unique/ circulating recombinant may play an increasing role in the HIV pandemic. The review article published from Africa on HIV-1 diversity, demonstrated that there is a high diversity of HIV-1 in west-central Africa. A few subtypes namely A, C, CRFO2_AG and D accounted for about 85% of new infections. Subtype A and D were stable in East Africa; C in Southern Africa; A, G, CRFO2_AG and CRFO6_cpx in Western Africa; and subtype B and CRFO2_AG in northern Africa [Rapheal W. Lihana *et al.*, 2012]

HIV-1 Diversity in infants

The time between HIV detection and initiation of ART in children should be less for effective implementation of therapy. A study in rural Zambia reveals various issues for delays in ART initiation in children which needs to address for effective earlier treatment [Sutcliffe CG.et.al.2016]. The effective implementation of early ART treatment restricts the viral evolution. The new viral variants can be avoided by early ART treatment. Studies on genetic diversity may have important role in host immune control to achieve viral reduction by implementing customization in therapeutic approaches case to case basis [Palma P.et.al.2016]

Among HIV exposed children with high risk of morbidity and mortality, complimentary feeding plays a very important role for refining survival of child and endorsing growth and development. In the low income countries, nutrition program is highly recommended in respect with best complimentary feeding and dilatory diversity to improve the nutritional status [Kamenju P.et.al.2017]. Children born to HIV infected mother showed a high prevalence of HIV drug resistance mutation with specially Transmitted Drug Resistance. This prevalence hampers the effectiveness of ART. HIV drug resistance Surveillance is only way to implement ART program without any obstacles [Andrade SD.et.al.2017]

Scarcity of information regarding HIV-1, transmitted founder (TF) virus and massive genetic diversity, are the most common obstacles for HIV vaccine development. The study on characterization with identification of salient features of transmitted founder (TF) virus reveals lower sensitivity of TF variants to maraviroc than the virus variants in adults with chronic HIV infection [Ashokkumar M.et.al.2018].

In Africa, the first CRF 47-BF was identified and transmission cluster where there was increase of subtype A and subtype F1 and reduction of subtype A1 and sub type D were recognized. The current information related to subtypes, CRF, URF among children and

adolescents helps to plan future program management in connection with HIV-AIDS [Rubio-Garrido M.et.al.2020]. HIV-1 subtype diversity in a population always represents local epidemics. A study in Australia reports non-B origin females in heterosexual transmission where subtype B infections were associated with clusters and singletons infections were identified in non-B infections. Over the time, the changes in HIV epidemic were reported with respect to demographic associations of HIV subtypes [Di Giallonardo F.et.al.2020]. As the maternal ART fails to eliminate burden of mother to child transmission, a maternal active immunization strategy studied to reduce the load of mother to child transmission. Two envelope vaccine trials showed no ability to enhance and neutralize autologous virus, the necessity of an alternative approaches come to obstruct vertical transmission by getting neutralizing and nonneutralizing antibody responses that represents broad diversity of HIV strains [Hompe ED.et.al.2020].

In HIV transmission, maternal antibodies transferred to their infants at the time of viral exposure although 100% infants are not infected regardless of continued exposure to virus. For HIV vaccine designing, understanding the HIV specific immune responses plays most important role. Studies on HIV specific antibodies characterization and transmitted virus in infants will have significant contribution in HIV management [Doepker LE.et.al. 2020]

HIV Drug resistance

Combination antiretroviral therapy can suppress HIV-1 replication to undetectable levels with concomitant significant clinical outcomes. However, suboptimal suppression HIV-1 replication can result in the emergence of drug resistant virus strains. HIV-1 isolates that have acquired mutations conferring reduced susceptibility to antiretroviral drugs can be can be transmitted, potentially limiting options for first line therapy in untreated individuals [Shafer RW.et. al. 2008]

HIV-1 has a high mutation rate, accumulating nearly one nucleotide mutation per replication cycle [Mansky LM. et al. 2002 & Abram ME.et. al.2010] Although individuals are usually infected with only a single or few original clones, an estimated 10^{10} virions are produced each day in untreated individuals, resulting in innumerable virus variants, often called a quasispecies [Perelson AS *et al.*, 1996 & Coffin JM 1995].

The proportion of patients without prior antiretroviral therapy and who are infected with a virus resistant to at least one antiretroviral drug in Australia, Europe, Japan and the United States of America is 10% to 17%, while data between 2006 and 2010 suggests that transmitted antiretroviral drug resistance among those starting antiretroviral treatment in low- and middleincome countries increasing [UNAIDS: The gap report. Geneva 2014].

The study conducted at South Africa where the participants were from an antiretroviral treatment naïve cohort, some antiretroviral drug mutations were detected (Table 4). The NNRTI mutation K103N detected on the 0143A sequence causes highlevel resistance to nevirapine (NVP), and efavirenz (EFV). The NNRTI mutation, E138A, detected on the 0143A sequence is a polymorphism that may contribute to reduced etravirine (ETR) and rilpivirine (RPV) susceptibility in combination with other NNRTI-resistance mutations. The K101E mutation found on the 0189A sequence causes intermediate resistance to NVP and low level resistance to EFV, ETR, and RPV. No major PI mutations were detected in the study [Msimanga P. W. et.al.2015].

Transmitted and Acquired Drug Resistant

The ability of HIV-1 virus to rapidly generate new variants allows HIV-1 to evade the immune system and fosters the development of ARV drug resistance. In fact, the development of antiviral resistance is considered essential to proving that an ARV candidate compound inhibits HIV-1 directly, rather than the host cells in which the virus replicates.

HIV-1 drug-resistance mutations occur at the target of therapy and, almost without exception, decrease viral replication fitness. Drug resistance can either be acquired through drug selection pressure (acquired resistance), or transmitted from person to person (transmitted resistance). Naturally occurring drug-resistant viruses (i.e. resistance in viruses that have not been subject to selective drug pressure) are rare. As an important consequence, most drug-resistance mutations are non-polymorphic, meaning that they are rarely observed in un-treated patients [Michele W *et. al.*, 2012].

Although drug-resistant mutations usually re-duce viral fitness, most transmitted drug-resistant viruses revert to wild type gradually over a period of several years [Pao D et al. 2004 & Wittkop L, et al., 2011]. This slow reversion occurs because most new infections are monoclonal and during the expansion and diversification of this clone early in infection, it acquires multiple cytotoxic T-lymphocyte (CTL)-escape mutations, which may provide the drug-resistant virus a temporary selective advantage against wild-type viral revertants [Goonetilleke N *et al*., 2009].

There is essentially no cross-resistance between drug classes. Even viruses with high levels of resistance to drugs in one ARV class are fully susceptible to drugs belonging to a previously unused ARV class. In the case of the NRTIs and NNRTIs, both of which inhibit reverse transcriptase, there is often in vitro synergism, in that NRTI-resistant viruses often increase NNRTI susceptibility and NNRTI-resistant viruses occasionally increase NRTI susceptibility [Whitcomb JM et al., 2002 & Basavapathruni A *et. al.,* 2006].

The study conducted at United States showed that Transmitted drug resistance (TDR), the primary acquisition of an HIV variant already resistant to antiretroviral, impacts approximately 15% of all new infections in the United States. Historically, from the time

initial agents in the reverse transcriptase, protease, and entry inhibitor classes were introduced [Christopher B. Hurt 2011].

A retrospective analysis of susceptibility to antiretroviral drugs before treatment and drug-resistance mutations in HIV in plasma samples from 377 subjects with primary HIV infection who had not yet received treatment was performed in North American cities. Initial antiretroviral therapy is more likely to fail in patients who are infected with drug-resistant virus [Little SJ et.al. 2002].

The TDR reported from Western Kenya from mother to child was reported in 29_67% and in adults among 1.1_7.5% in the coast and Nairobi. Resistance upon ART failure has only been reported in three studies, one describing 14% resistance in 58 injecting drug users in Mombasa with no available treatment histories [Kantor R. et. al. 2014]

The study conducted in a middle income country at Brazil during 2002 to 2012 on trends and predictors of HIV-1 acquired drug resistance revels mutation rate decrease for PI, NRTI, IAS-3 class mutations from 69.2% to 20.7%, 92.3% to 90.2%, 46.2% to 22.5%, and 12.8% to 5.7%, respectively ($p<0.05$) whereas increase in NNRTI resistance observed from 74.4% to 81.6%. Increase in resistance testing and availability of more therapeutic option may support the declining trends [Duani H. et al. 2016]. The risk of acquired HIV drug resistance is very low unless immediate ART initiation was observed in a cohort study of HIV CAUSAL collaboration among 50981 recruited individuals [Lodi S. et. al. 2018]. A study conducted at Tanzania on Pre-treatment and Acquired HIV drug resistance indicated mutations among ART naïve individual whereas 14.9% mutation with high level mutations with drug of first line regimen. Patients with multiple drug resistance to all available ART regimens were observed in this study. High prevalence rate of pre-treatment and acquired HIV drug resistance mutation hamper the efficacy of ART program at Tanzania [Barabona G. et. al.

2019]. A study among HIV infected women from a high risk cohort in Uganda shows high levels of acquired HIV drug resistance following virological non suppression where acquired HIV drug resistance to NNRTIs and NRTIs were detected 81.3% and 45.8% respectively. Shifting to ART timely and proper adherence in counseling is highly recommended when moderate viral suppression and high HIV drug resistance prevalence was observed in cohort [Seguija F. et al. 2020]. A nationally representative survey was conducted in Honduras between 2016-2017 on high level of pre-treatment and acquired HIV drug resistance, where viral load suppression rate was 89.7% in ADR12 and 67.9% in ADR48 to any drug among PLHIV with VL ≥ 1000 copies/ml. Non-NNRTI based first line regimen was strongly recommended for ART initiation as high prevalence of NNRTI pre-treatment HIV drug resistance was observed in this survey [Girón-Callejas A. et al 2020]

A study on estimating the prevalence HIV drug resistance with an innovative idea for resource poor countries by using pooled samples revealed decrease in surveillance cost by presenting Bayesian model for pool testing data. When a non-model-based estimator failed, the simulation study conducted in this study recommends the pool size which reduced the prevalence parameter identifiable in occasions [Finucane MM et. al. 2016]. Low rates of transmitted HIV drug resistance after initiation of ART for 10 years also supports TDR surveillance system as public health priority. Subtype diversity of HIV-1 shows significant variation with seroconverters with infection of wild type virus without Transmitted drug resistance. Surveillance among known seroconverters to estimate transmitted drug resistance is recommended [Reynolds SJ. et. al. 2017]. Transmitted Drug Resistance reported to protease inhibitors (PIs), 1.3% having TDR to nucleoside reverse transcriptase inhibitors (NRTIs), and 1.6% to non-nucleoside reverse transcriptase inhibitors (NNRTIs) in a cross-sectional study conducted at China where no significance difference was found in prevalence

of TDR in compare with nationwide survey although in phylogenetic trees, four drug-resistant transmission clusters were recognized [Zhao S.et.al.2018].

Integrated HIV surveillance along with primary drug resistance is highly recommended whereas resistance to NRTI and NNRTI were new challenge for newly infected cases. Introduction of testing rapidly and improvement of treatment policy with easy ART accessibility are also suggested for integrated surveillance system [Chauhan CK. et. al. 2019]. Before starting of ART to HIV positive subjects, drug resistance testing is highly recommended in HIV treatment guidelines where resistance testing to reverse transcriptase inhibitors and PIs are most common in compare with integrase strand transfer inhibitor (InSTI) that is not consistent. In adults with recent HIV-1 infection, InSTI is uncommon although it is suggested to inform clinical practice in surveillance study [Mbisa JL. et.al 2020]. Genetic diversity of HIV-1 is not constant and that results increase of non-B subtypes significantly. Genotypic resistance testing (GRT) before starting ART is clearly suggested when detection of INSTI mutation detected from a single center study. Continuous surveillance of resistance mutations is only way for early detection of HIV drug resistance mutations where there was no significance reduction of TDR was found [Mazzuti L. et. al. 2020].

Virological failure

CD4 and viral load testing will be assayed following standard protocol. Virological failure and detectable viral load are defined as VL > 400 copies/ ml.

The transmission of drug-resistant HIV-1 strains is associated with a high risk of virological failure. Although patients infected with a virus containing one or more transmitted drug-resistance mutations are likely to be at higher risk of harbouring additional minority drug-resistant variants, the virological response to a regimen selected based on the results of

standard geno-typic testing appears to be almost as effective as the initial treatment of a patient without transmitted resistance [Wittkop L *et al.*, 2011 & Chaix ML,]

Despite the equivalent efficacy and more favorable resistance implications of PI- versus NNRTI-based first line therapy, widespread use of PI-based first-line therapy is not warranted at this time, due to resource limitations and predicted increased risk of resistance-related failure of NNRTI/NRTI second-line regimens. PI-based first-line therapy could be reconsidered when antiretroviral agents from other classes become available for second-line regimens in resource-limited settings [Hill A et al., 2013].

Immunological monitoring as a sole indicator of virological failure would lead to a premature switch to valuable second-line regimens for 58% of patients who experience a 25% decrease in CD4 cell count and for 43% patients who experience a 50% decrease in CD4 cell count. Selective virological monitoring and the addition of indicators like trends CD4% percent decrease and duration of therapy may systematically improve the identification of treatment failure. VL testing is now mandatory for patients suspected of experiencing first-line treatment failure within the Academic Model Providing Access to Healthcare (AMPATH) in western Kenya, and should be considered in all resource-limited settings [Kantor R et al., 2009].

In patients developing virological failure on first-line ART, the extent of drug resistance is roughly proportional to the duration of uncontrolled virus replication in the face of selected drug pressure. In resource-limited countries where patients undergo infrequent virological monitoring, samples from patients with virological failure generally contain more drug-resistance mutations and higher levels of cross-resistance than virological samples from patients with virological failure in well-resourced regions [Gupta R, et al., 2008].

To improve initial treatment success, easy access to antiretroviral is highly recommended where first ART regimen was modified among patients and risk of subsequent modification, mortality and virologic failure were high [Wolff M. et. al. 2016]. Where the antiretroviral drugs are widely used, the prevention and control strategy of HIV should be based on epidemic characteristics of HIV-1-resistant strains [Lu X, Zhao H. et. al. 2017]. CD4 cell count for detection of treatment failure results delayed in shifting to second line ART. In addition to that the study revealed that, for detection of treatment failure, WHO immunological criteria had low sensitivity and positive predictive value [Gunda DW. et. al. 2017]. Association of pre-ART and virologic failure among adults was studied where a positive association found between higher pre-ART pol diversity and the risk of virologic failure in adults in compare to contrary relationship reported previously in children [Kearney MF. et. al. 2018]. The study at five hospital in Haiti displayed viriologic suppression among two third of patients after receiving ART for six months. HIV-1 viral load testing in robust way was highly recommended for monitoring ART outcomes [Jean Louis F. et. al. 2018]. A cross sectional study was carried out at Uganda on virologic failure in HIV positive adolescent with perfect adherence exposed that virologic failure were observed in significantly adherent (adherence >95) adolescents. Adolescents with unsuppressed viral loads experienced virologic failure robustly. Switching to drug regimen with timely counseling was highly recommended for adolescent in this study [Natukunda J. et. al. 2019]. A study at a tertiary care hospital in Ethiopia was conducted on Predictors of treatment failure, time to switch and reasons for switching to second line antiretroviral therapy in HIV infected children receiving first line anti-retroviral therapy showed predictors of treatment failure like negligence by parents, negative sero status caretakers and initiation of cART at younger age [Haile GS. et. al. 2019]. To modify the ART guidelines and to understand HIV-1 epidemiology, surveillance of virological failure, HIV-1 subtypes and drug resistance played

important role. Virological failure, drug resistance and overall HIV-1 transmission can be reduced by formatting strategies based on the population [Yuan D. et. al. 2020]. A study exposed the prevalence of HIV drug resistance found relatively low among treated patients. To understand HIV-1 subtypes and HIV-1 drug resistance prevalence, HIVDR surveillance played an important role to improve clinical management, prevention of HIV [Chen J. et. al. 2020]

Implication of HIV diversity for HIV therapy

The high diversity of HIV and the high turnover of virus help to spawn resistance to antiretroviral drugs. That is why single-drug regimens such as the early zidovudine trials in the 1980s caused an immediate sharp fall in viral load, but led to the emergence of resistant strains within a few weeks of initiating treatment. It was not until the introduction of triple drug antiretroviral therapy (ART) in 1996 that a long-lasting clinical benefit became apparent. This has revolutionized therapeutic schedules for HIV infection [Vella S *et al*. 2012].

Chapter 2

HIV-1 Diversity among ART naïve HIV infected individual

Preamble

India having the third major HIV epidemic on earth with an estimate of 2.1 million people are living with HIV (PLHIV) along with more than sixty-five thousand deaths in past years [WHO, UNAIDS, UNICEF. Global HIV/AIDS response: epidemic update and health sector progress towards universal access; 2015]. Transmission of HIV with anti-retroviral (ARV) drug resistance mutations (DRMs) has a negative impact on prevention of HIV through ART. High active antiretroviral therapy is leading to the reduction of mortality [Bhaskaran K.et.al.2008; Jahn A.et.al.2008]. However, ART might be the causative agent behind in rise of drug-resistant HIV strains [Ives NJ.et.al.2001; Mills EJ.et.al.2011]. Approximately 10% of PLHIV in ART develop drug resistance to the antiretroviral (ARV) drugs within two years [6, 7, 8]. Generally, failure of ART occurs due to drug resistance mutation, pre-therapy resistance mutations, suboptimal potency, and wrongly prescribed antiretroviral drugs by physicians [Schwartländer B.et.al.2011]. Rise of drug resistance mutations in HIV along with increasing use of antiretroviral drugs not only commenced with the treatment failures but developed a new phenomenon that are people with pre-treatment drug resistance to HIV (PPDRH) [Xu, X.et.al.2021; Hosseinipour MC.et.al.2009]. Pre-treatment drug resistance or PDR is that drug resistance has been recognized by diagnose the individual's resistance to ARV drugs preceding to antiretroviral therapy [Leger P.et.al.2009; Hamers R.et.al.2011]. Several new articles revealed the jeopardy of virologic failure within fifty weeks of antiretroviral treatment in PPDRH, it is two to three times higher, in dipping the enduring effectiveness of ARV drugs [Gupta R.et.al.2008]. Polymorphisms of HIV polymerase gene have previously been reported to be related with DRM in HIV-1, B (subtype). It was also found that HIV-1 subtype C is predominant in India [Deshpande A.et.al.2004; Hira SK.et.al.2004]. HIV DRMs described in India are as Nucleoside Reverse Transcriptase Inhibitor (NRTI) mutation by the thymidine analogue mutations (TAMs), Non-Nucleoside

Reverse Transcriptase Inhibitor (NNRTI) mutation and Protease Inhibitor (PI) mutation [Lakshmi R.et.al.2009]. Presence of DRM in people with pre-treatment drug resistance to HIV (PPDRH), has implications for therapeutic options and outcomes. But, information on HIVDR mutations among PPDRH from eastern India are limited [Lakshmi R.et.al.2010]. Continued success of ART requires appropriate program planning based on HIV drug resistance mutation patterns. This study aimed to generate evidence on the status of people with pre-treatment drug resistance to HIV from Kolkata a metro city having a long inheritance of cultural heritage form eastern India.

Materials and Methods

Study Setting

The study was carried out at Integrated Counseling and Testing Center (ICTC) of ICMR-National Institute of Cholera and Enteric Diseases which is accredited by NABL and maintains international standard of ISO 15189:2012 to provide quality medical laboratory service.

Adult (age > 18 years) first time attendee of ICTC, ICMR-NICED, irrespective of sex, ethnicity, education or occupation were enrolled for present cross-sectional study. The study was conducted during April 2017 to March 2020. A total of 1340 ICTC attendee during this period are recruited for the present study following informed consent. The study was approved by Institutional Ethical Committee.

Collection and Processing of Sample

Informed consent was administered in front of an experienced Counselor and about 5 ml blood was drawn from consenting participants by competent phlebotomist at ICTC. Plasma was separated within 6 hours, aliquoted in 1ml volume and stored in cryo-vials, then frozen at

−70°C for further processing. Every time fresh aliquote was used following the methods of resistance testing guidelines published independently by HIVResNet Laboratory Working Group and WHO [Eshleman H.et.al.2004; http://hivdb.stanford.edu; Bennett DE.et.al.2009].

Data Collection:

Elementary socio-demographic data (age, sex, ethnic, education, marital status, and occupation) with behavioral characteristics (route of HIV infection etc.) were collected through an approved set of previously prepared questionnaires by an experienced Counsellor.

HIV-1 Sero-positivity status

All the participants were screened for HIV-1 sero-positivity followed by three tests strategy of NACO with three different principles that are Dot Immunoassay, Immunochromatography & immunoconcentration [www.nacoonline.org]. Among these 1340 participants, total 104 detected as HIV-1 positive and proceed for quantification of viral load and genotyping of HIV-1.

Viral load Estimation

The Abbott Real Time HIV-1 assay uses RT-PCR[26] to generate amplified product from the RNA genome of HIV-1 in clinical specimens. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labelled oligonucleotide probes on the Abbott *m*2000*rt* instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the

Abbott *m*2000*rt* is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Procedure

1. Assay controls and IC thawed at 15 to 30°C or at 2 to 8°C.
2. Calibrators were also thawed at 15 to 30°C or at 2 to 8°C only if performing a calibration run following manufacturer's instructions.
3. The Controls were vortexes for 3 times for 2 to 3 seconds before use. Ensure that the contents of each vial are at the bottom after vortexing by tapping the vials on the bench to bring liquid to the bottom of the vial.
4. Amplification reagents thawed at 15 to 30°C or at 2 to 8°C and store at 2 to 8°C until required for the amplification master mix procedure.
5. Abbott *m*Sample Preparation bottles were inverted gently to ensure a homogeneous solution. If crystals are observed in any of the reagent bottles upon opening, the reagents were allowed to equilibrate at room temperature until the crystals disappear.
6. IC was vortexed 3 times for 2 to 3 seconds before use.
7. A calibrated precision pipette was used to add 500 µL of IC to each bottle of *m*Lysis Buffer. And mixing was done by gently inverting the container 5 to 10 times to minimize foaming.
8. A total of 96 samples can be processed in each run, with the exception of the 1.0 ml Assay Application. A negative control, a low positive control, and a high positive control are included in each run, therefore allowing a maximum of 93 specimens to be processed per run.

9. Low and high positive controls, negative controls and the calibrators (if applicable) and the patient specimens were placed into the Abbott *m*2000*sp* sample rack.
10. The 5ml Reaction Vessels were placed into the Abbott *m*2000*sp* 1 ml subsystem carrier.
11. Abbott *m*Sample preparation System reagents and the Abbott 96 Deep- well Plate were loaded on the Abbott *m*2000*sp* worktable as described in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.
12. From the Protocol screen, appropriate application file was selected corresponding to the sample volume being tested.
13. Sample extraction protocol was initiated as described in the Abbott *m*2000*sp* Operations Manual, Operating Instruction section.
14. The Abbott *m*2000*sp* Master Mix Addition protocol needs to be initiated within 1 hour after completion of Sample Preparation.
15. Amplification reagents and the master mix vial were loaded on the Abbott *m*2000*sp* worktable after sample preparation is completed.
    - Each Amplification Reagent Pack supports up to 24 reactions.
    - Prior to opening the amplification reagents, ensure that the contents are at the bottom of the vials by tapping the vials in an upright position on the bench.
    - Amplification vial caps were removed and discarded.
    - A second Amplification Reagent Pack is required if performing 25 to 48 reactions.
    - A third Amplification Reagent Pack is required if performing 49 to 72 reactions.
    - A fourth Amplification Reagent Pack is required if performing 73 to 96 reactions.

16. Appropriate deep-well plate was selected that matches the corresponding sample preparation extraction. Initiate the Abbott *m*2000*sp* Master Mix Addition protocol. Instructions as described in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section were followed.

    - After sample extraction complete, the Abbott *m*2000*sp* automatically fills any empty wells in the Abbott 96-Well Optical Reaction Plate when there are greater than 48 samples processed within a run.
    - If prompted by the instrument, Reagent Carrier 2 should remain in place, minimally containing the reagent vessel for *m*Elution Buffer (Reagent Carrier 2, location 6). If this reagent vessel has been unloaded, a new reagent vessel with the *m*Elution Buffer label into Reagent Carrier 2, location 6 was placed. System fluid was added to the reagent vessel and used to fill empty wells. Once this process is complete, the system will continue with the master mix addition.
    - Pure Dye optical re-calibration of the Abbott Real Time HIV-1 assay- specific dyes (FAM, VIC, or ROX) is performed per the Calibration Procedures section of the Abbott *m*2000*rt* Operations Manual.

17. Abbott *m*2000*rt* instrument was switched on and initialized in the Amplification Area.
18. Abbott 96-Well Optical Reaction Plate was sealed after the Abbott *m*2000*sp* instrument has completed addition of samples and master mix according to the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.
19. Sealed optical reaction plate was placed into the Abbott Splash-Free Support Base for transfer to the Abbott *m*2000*rt* instrument.
20. Abbott 96-Well Optical Reaction Plate was placed in the Abbott *m*2000*rt* instrument. From the Protocol screen, appropriate application file corresponding to the sample

volume being tested was selected. Abbott Real Time HIV-1 protocol was initiated, as described in the Abbott *m*2000*rt* Operations Manual, Operating Instructions section.

Post Processing Procedure: Abbott 96 Deep-Well Plate was removed from the worktable and dispose of according to the Abbott *m*2000*sp* Operations Manual.

HIV-1 Genotyping

QIAamp (Qiagen, GmH, Hilden, Germany) kits were used for RNA extraction, purification, amplification and Big Dye chemistry was used for sequencing of PCR products.

(i) RNA Extraction and Purification

- Extraction of RNA and purification has been done by using QIAamp Viral RNA mini kit followed by manufacturer's instruction.
- QIAamp Viral RNA Mini Kits represents a well-established technology for general use viral RNA preparation. The kit combines the selective binding properties of a silica based membrane with the speed of microspin.
- Lysis: The sample is first lysed under the highly denaturing conditions provided by buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to buffer AVL, improves the binding of viral RNA to the QIAamp membrane.
- Then the sample was loaded onto the QIAamp Mini Spin column.
- The RNA binds to the membrane, and contaminants efficiently washed away in 2 steps using 2 different wash buffer.
- High quality RNA is eluted in a special RNase free buffer, ready for direct use or safe storage.
- The purified RNA is free of protein, nucleases and other contaminants and inhibitors.

(ii) Amplification

RT-PCR

HIV-1 pol gene was amplified by one tube reverse transcriptase Polymerase Chain Reaction (RT-PCR). The total procedure has been carried out by using Qiagen RT-PR Mini kit (Qiagen, GmBH, Hilden, Germany) manufacturer's instructions.

The procedure was carried out by round -1 RT-PCR followed by nested PCR.

RT-PCR master mix is prepared followed by proper vortexing and centrifugation as per standard protocol described in the manufacturer's instructions.

Table 2.1: Preparation of RT – PCR master mix

Reagent	Volume 1 rxn (µl)
RNase – free water	5.5
5x Qiagen One Step RT-PCR buffer (Contains 12.5 mM $MgCl_2$)	5.0
2021 F Primer (10 pmol/ µl)	1.0
4521 R Primer (10 pmol/ µl)	1.0
Dntp mix (10mM each)	1.0
RNAse inhibitor (20U/µl)	0.5
Qiagen One Step RT-PCR Enzyme mix	1.0
Total Vol. (µl)	15
RNA Template	10
Master mix was vortexed for 3-5 seconds in low speed. To bring the contents in bottom of the 1.5 ml mirocentrifuge tube, centrifugation was done.	

In 200 µl MicroAmp reaction tube, 10 µl of RNA extract was added and similarly addition of positive and negative control was done.

The following program was carried out in the thermal cycler.

Table 2.2: Thermo Cycling profile for initiation of RT-PCR

Program	Temperature	Time	Process
A	65ºC	30 seconds	Relax and denature RA secondary structure
B	42 ºC	5 minutes	Cool to optimal enzyme activity temperature
Program A and B were run and thermal cycler was paused.			

Inside the laminar airflow, 15 µl RT-PCR brewmix was added to each tube. The RT-PCR master mix contains RNase free water, 5x Qiagen One Step RT-PR buffer (contains 12.5mM $MgCl_2$), 2021 F primer, 4521 R primer, dNTP mix, RNAse inhibitor, QIAGEN One step RT PCR enzyme mix, RNA Template.

The thermal cycler was turned on and QIA-RT-PCR program was run.

The thermal cycling profile for RT-PCR and Nested PCR were tabulated in Table 2 and Table 3.

Table 2.3: Thermo cycling profile for QIA-RT-PCR

Number of cycles	Temperature	Time	Process
1	50 ºC	30 minutes	The first strand cDNA synthesis was carried out
1	95 ºC	15 minutes	*HotStarTaq* DNA Polymerase is activated by this heating step. Omniscript and Sensiscript Reverse Transcriptasesare inactivated and the cDNA template is denatured.
35	94 ºC	20 seconds	The second strand synthesis and PCR amplification.
	59 ºC	45 seconds	
	72 ºC	3 minutes	
1	72 ºC	10 minutes	Final Extension

Nested – PCR Amplification:

- In a 1.5 ml micro centrifuge tube, the following reagents were added in order: Taq DNA polymerase (Genei, Bangalore, India), 3 U/ µl (1 µl) in a 10x PCR buffer B (5 µl), 25 mM $MgCl_2$ (4 µl), 2mM dNTPs Mix (5 µl), 10 pmol of each primer and 6 µl of RT-PCR product, The final volume of 50 µl reaction was made up by using DNase / RNase free water.

Table 2.4: Preparation of Nested – PCR master mix

Reagent	Volume 1 sample (µl)
10X PCR buffer B	5
2mM dNTPs mix	5
25 mM $MgCl_2$	4
2135 F (10 pmol)	2
3338 R (10 pmol)	2
Taq DNA Polymerase 3 U/ µl	1
DNase-RNase free water	25
Master mix was vortexed for 3-5 seconds in low speed. To bring the contents in bottom of the 1.5 ml mirocentrifuge tube, centrifugation was done.	

- After centrifugation, 44 µl of PCR master mix was pipetted out and 6 µl of RT-PCR product was added to Micro Amp sample tube.

- Nested-PCR reaction was performed in the thermal cycler using the prescribed program.

Table 2.5: Thermo cycling profile for Nested-PCR

Number of cycles	Temperature	Time	Process
1	94 ºC	2 minutes	Initial Denaturation
25	94 ºC	20 seconds	Denatures NA
	59 ºC	45 seconds	Anneals primers
	72 ºC	3 minutes	Extends DNA
1	72 ºC	10 minutes	Final Extension
----	4 ºC	hold	Keeps samples cold

Table 2.6: Primer used for amplification protease and RT region of HIV-1

Primer	Sequence
2021 F	5'- AAG GCT GTT GGA AAT GTG G -3'
4521 R	5'- RCT GTT TCT TGT CCT GTT TCT GC -3'
2135 F	5'- TTT AGA GCA GAC CAG AGC CAA CAG C -3'
3338 R	5'- TTT TCC CAC TAA CTT CTG TAT AGT CAT TG -3'

(iii) Gel Electrophoresis and purification

5 - 10 µl nested-PCR products were checked by electrophoresis at 100V on 1% agarose gel in 1X TAE buffer along with standard molecular weightmarker. Ethidium bromide solution was used as staining solution for 30 mins and visualized under UV-illuminator.. The PCR product was purified by using commercially available PCR purification kit.

(iv) Sequencing PCR Product:

- Before starting the procedure, the purified PCR product was diluted to 30 ng/µl (15 – 25 µl total volume) using DEPC treated water.

- The sequencing PCR was done by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystem, CA, USA). The Big Dye chemistry was carried out with HIV-SEQ mix and diluted purified PCR product with final reaction volume 10 µl

Table 2.7: Preparation of In-house HIV-SEQ Mix for each sequencing primer

Reagents	Volume
Big Dye Terminator Ready Reaction Mix	2µl
5x sequencing buffer	2µl
Primer (2pmoles/2µl)	2µl
DNase, RNase free water	2µl
HIV-SEQ mix was prepared for each sequencing primer	

- Diluted, purified PCR products and the sequencing primers were thawed.
- HIV Seq mixes was pulse vortexed.
- To the appropriate well of the plate, 8 µl HIV Seq mix were added followed by necessary precautions as per manufacturer's instruction.
- PCR product was vortexed. 2µl diluted, purified PCR product was added to the well into the plate.
- Centrifugation and spinning was done as per manufacturer's instruction
- Cover the plate with a rubber 96 well plate septa.
- Incubate sequencing Reactions in the thermal cycler using following program

Table 2.8: Thermo cycling profile for Sequencing PCR (Big Dye Chemistry)

Number of cycles	Temperature	Time
25	96 ºC	10 Second
	50 ºC	5 second
	60 ºC	4 minutes
--	4 ºC	hold

Table 2.9: Primer used for HIV-1 Sequencing PCR

Primer	**Sequence**
2135 F	5'- TTT AGAGCAGACCAGAGCCAACAG C -3'
2493 F	5'- CCT GTC AAC ATA ATT GGA AG -3'
3012 F	5'- GGA TCA CCA GCA ATA TTC -3'
2557 R	5'- GGT ACA GTT TCA ATG GGA C -3'
3117 R	5'- CCC TAT TTC TAA GTC AGA TCC -3'
3338 R	5'- TTT TCC CAC TAA CTT CTG TAT AGT CAT TG -3'
3403 F	5'- GGG CCA AAG TAC TAA CAG -3'
3768 F	5'- CAA GCC ACC TGG ATT CCT GAG TG -3'
3999 R	5'- CCT GAA TCT TGC AAA GCT AG -3'
4381 R	5'- CCT GGA CTA CAG TCT ACT TGT CCA T-3'

(v) Post Sequencing PCR Precipitation:

- Using 200µl multichannel pipettor, 80µl of 80% isopropanol to each 10 µl sequencing reaction was added.

- The plate was covered immediately with septa
- Mixing was done by inverting the plate three times.
- The plate was incubated for 15minutes at room temperature in dark.
- Centrifugation was done for 45 minutes at 35R at 2000 x g
- After removing the cover, the plate was inverted on a folded tissue paper and centrifugation was done at 700 g x for 1 minute.
- The plate was dried for 5 minutes
- The samples were resuspended in 20µl of HiDi Formamide cover with septa.
- Finally, the plate was loaded into the sequencer for sequencing.

(vi) Analysis

ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA): The PCR products were sequenced on a 16 capillary automated florescence based Genetic Analyzer. ABI data collection software was used to program the sequence analysis file. The Sequence Analysis Software v5.3 was used to extract base calling sequenced data.

Sequence Analysis: The ABI files for each sample were analyzed by using ABI Sequence Analysis Software v5.3.

The forward and reverse sequences for each sample were assembled and edited with wild type reference sequence HXB-2 where multiple sequence alignment Secscape software was used and the FASTA sequences were exported.

HIV Drug Resistance Algorithm: The obtained DNA sequences were submitted to HIV drug resistance database of Stanford University (http://hivdb.stanford.edu/hiv). Subtyping of

obtained sequences was performed through comparing them with reference sequence through Basic Local Alignment Search Tool (BLAST).

Quality control

For quality control of HIV-1 genotyping, negative, low-positive, and high-positive control samples were run with every batch. The positive controls ensured the RT-PCR and genotyping success. To ensure good sequence quality, the high-positive control was sequenced before genotyping the HIV-1 clinical samples, precluding editing mistakes.

Clade typing

HIV-1 subtype was defined using the REGA HIV-1 sub-typing tool from the Stanford HIV drug-resistance database (http://hivdb.stanford.edu/). Worldwide subtype references were obtained from the Los Alamos HIV database. Nucleoside reverse transcriptase inhibitors (NRTI) mutations and Non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations for RT gene sequences were mostly included in the present study.

Statistical analysis

Data was first recorded on a predesigned paper form and subsequently transferred to a Microsoft Excel spreadsheet for statistical analysis. All the entries were checked for possible keyboard error(s) at the entry level. Baseline clinical and biological characters of the study subjects were summarized as frequency (%) for the categorical variables, and mean ± standard deviation (SD) for quantitative variables. Correlations also performed to find out the resistance patterns in these drugs.

Phylogenetic tree

Clade typing and phylogenetic reconstructions were performed using the REGA sub-typing tool of the HIV drug resistance database, and nucleotide sequences were aligned using the Clustal W multiple sequence alignment program. Phylogenetic analysis was conducted using MEGA v6.0 software. The neighbour-joining method and Kimura parameter model were used for tree construction with reliability estimated from 1000 bootstrap replicates [Thompson JD.et.al.1994; Kumar S.et.al.2004]. Simplot version 2.5, which is an interactive software program for Microsoft Windows computers, was used for identification of recombinants [Ray, S. C.et.al 1998]. The percent identity of the query sequence to a panel of reference sequences were calculated and plotted by SimPlot [KLole S. K.et.al.1999].

Results

All 1340 first time attendee of ICTC were recruited for present study. Among them 667 (49.77%) were male and 654 (48.8%) were female. A total number of 19 (1.41%) observed in other gender. The mean age of the study participants was 35.6 years (21-67 years). Most of the attendees, that mean 1179 are heterosexual (87.98%) with a small population of 134 (10%) people are bisexual and 27 (2.01%) people are homosexual. Most of the above said attendees 511 (38.13%) are educated up to secondary level. Remaining 359 (26.79%) people are having primary education, 299 (22.31%) people are having no education to people of 171 (12.76%) having education of graduation and more than graduation level. Among the people 841 (62.76%) are currently married or cohabiting, 287 (21.41%) people are belonging to separated/divorced/widowed and 212 (15.82%) people are never married. Most of the above said attendees that are 640 (47.76%) are non-employed to 320 (23.88%) are self-employed only 280 (20.89%) people are served to Government or private sectors (Table 2.10).

Table 2.10: Sociodemographic profile of first-time attendee of Integrated Counseling and Testing Centre (ICTC) of National Institute of Cholera and Enteric Diseases, Kolkata, between April, 2017 to march 2020 (n=1340).

Some important parameters along with statistical outcomes	
Age (yrs.) mean ± SD	44.2±5.24
Range	18-70
Gender	
Male	667
Female	654
other	19
Risk Factors	
Heterosexual	1179
Homosexual	27
Bi-sexual	134
Educational status	
Graduation and more	171
Secondary	511
Primary and self-literate	359
Illiterate	299
Marital status	
Married/cohabiting	841
Separated/ divorced/ widowed	287
Never married	212
Occupation	
Service	280
Self-employed	320
Non-employed	640

Among all 1340 subjects, 104 were tested positive for HIV-1 by three tests strategy of National AIDS Control Organization (NACO). CD4 estimation and RNA quantitation were performed for these 104 attendees. They were further evaluated through genome sequencing and genotyping (Table 2.11)

Table 2.11: Socio-demographic profile of first time ICTC (Integrated Counselling and Testing Centre of National Institute of Cholera and Enteric Diseases, Kolkata) attendee, tested and informed positive to HIV-1 and subjected for evaluation of pre-treatment drug resistance, between April, 2017 to march 2020 (n=104).

Some important parameters along with statistical outcomes	
Age (yrs.) mean ± SD	35.6±7.56
Range	21-65
Median CD4 cell count, cells/μL (IQR)	164 (134-217)
Median Viral load, log_{10} copies/mL (IQR)	4.97 (3.48-6.99)
BMI, Kg/m^2, mean ± SD	25.5±3.17
Range	14-36
Gender	
Male	66 (63.46 %)
Female	38 (36.54 %)
Risk Factors	
Heterosexual	97 (93.27 %)
Intravenous drug user	7 (6.73 %)
Locality	
Urban	7 (6.73 %)
Rural	97 (93.27 %)
Opportunistic infections, number (%)	
Cryptosporidiosis	2 (1.92 %)
Cytomegalovirus	13 (12.52 %)
Pulmonary TB	58 (55.76 %)
Extrapulmonary TB	22 (21.15 %)
Herpes Zoster	9 (8.65 %)

The result of genome sequencing and genotyping, 19 people (18.3 %) are found as pre-treatment drug resistance to HIV-1. Hence, the prevalence of pre-treatment drug resistance mutation for HIV-1 is 18.27%. The predominant route of transmission was heterosexual promiscuity as 17 (89.47%) subjects were counted for it and 2 (10.53%) people as intravenous drug users. All the obtained sequences were found subtype C. Two of them are

identified as recombinants of Subtype C. These two are submitted to Genebank and the accession number (LC570898 and LC529744) obtained. The phylogenetic tree prepared from the obtained sequences is shown as Fig. 2.1.

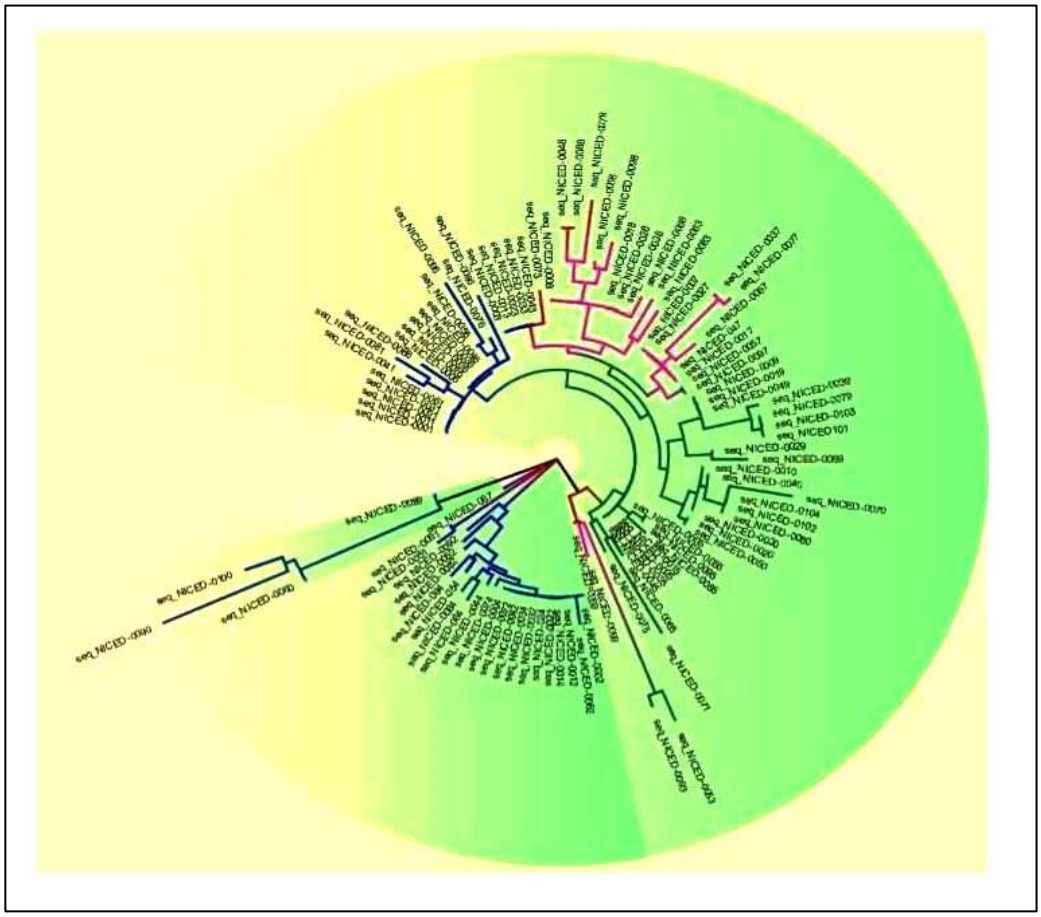

Figure 2.1: Molecular Phylogenetic analysis by Maximum Likelihood method: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3596.2987) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 104 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X.

Protease Inhibitor (PI) major drug resistance mutations detected were M46L (7.69 %) and V32I (0.96%). Whereas accessory PI drug resistance mutations were L23I & L24I (5.77%) and V82Deletion (0.96%). Nucleoside analog reverse-transcriptase inhibitors (NRTI) mutations for RT gene such as L74V (3.85%), M184V (16.35%), D67N (12.5%), K70N (7.69%), K70A (5.76%), K65Q (13.46%), D67S (12.5%), L74I (5.76%), M184R (10.58%), M184 Deletion (0.96%), T215 Deletion (4.81%), K65 Deletion (1.92%), D67DN (12.5%), L74K (1.92%), K65L (2.8%), M184R (10.6%), L74Y (0.96%) were detected and Non-nucleoside reverse-transcriptase inhibitors (NNRT) mutations such as E138EA (6.73%), E138R (0.96%), K101KE (8.65%), K103N (8.65%), G190GA (6.73%), V179D (5.76%), Y188L (5.76%), Y181C (7.69%), Y181 Deletion (0.96%), Y188 Deletion (0.96%), G190 Deletion (0.96%), Y181L (5.76%), Y188R (11.54%), G190G_REQRN (10.5%), Y188C (15.4%), K238T (7.69%) were detected (Fig. 2.2)

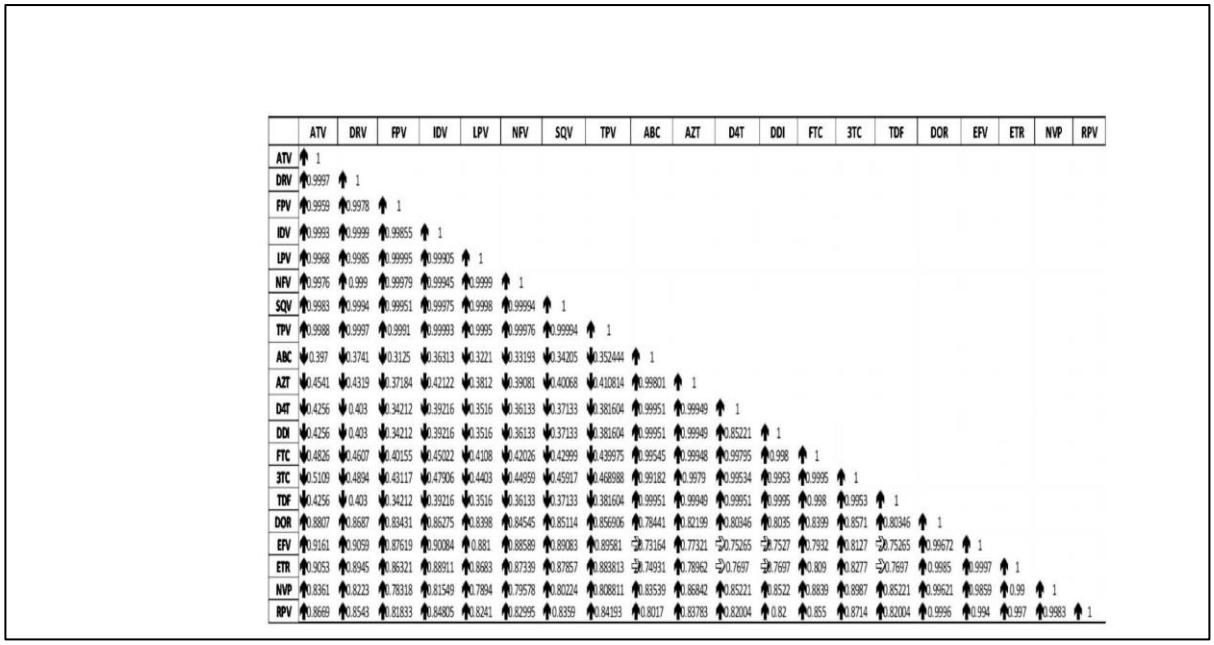

Figure 2.2: Correlation (P<0.05) matrix of resistance patters of drugs (protease inhibitors, nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors) among 104 treatment-naïve people living with HIV in Kolkata

TPV=Tipranavir; SQV=Saquinavir; NFV=Nelfinavir;

LPV=Lopinavir; IDV=Indinavir; FPV=Fosamprenavir; DRV=Darunavir; ATV=Atazanavir; TDF=Tenofovir; FTC=Emtricitabine; DDI=Didanosine; D4T=Stavudine;

AZT=Azidothymidine; ABC=Abacavir; 3TC=Lamivudine; RPV=Rilpivirine; NVP=Nevirapine; ETV=Etravirine; EFV=Efavirenz; ARVs Anti-retrovirals.

After the observation of correlation of resistance patterns of PI, it was revealed that ATV was highly correlated with, IDV, DRV, LPV FPV, NFV, SQV, and TPV. Similarly, a high correlation in the resistance patterns of DOR (0.8807), ETR (0.905), RPV (0.8669) and NFV (0.836) was also observed. In NRTIs, a very high correlation in pattern of resistance for ABC and DDI, DDI and D4T and D4T and AZT was found. Among NNRTIs, there was a high correlation in resistance patterns of NVP with other three NNRTIs (Fig. 2.3).

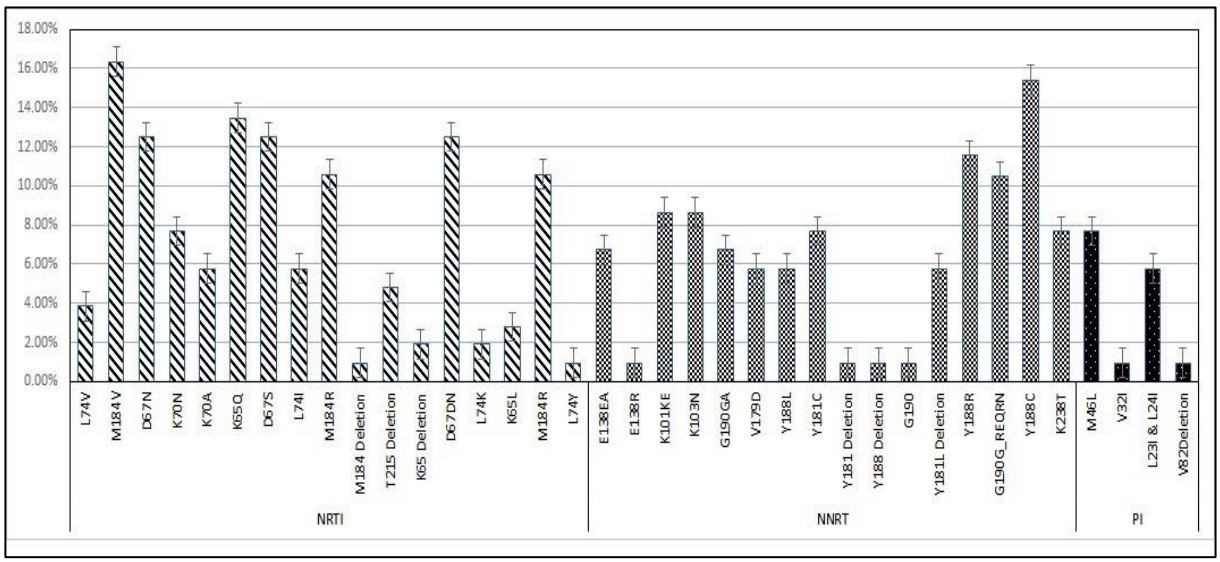

Figure 2.3: PDR (%) to antiretroviral drugs

The bootscan graph obtained through Simplot is shown in Fig. 4. Individual plot is analysed and compared between partial sequence of pol (1310 and 1289 nucleotides) and a reference pol region as seq_niced0025 and seq_niced0039. Individual point plotted is the percent

individuality within a sliding window widen up to 200 bp wide centred on the plotted location, with a step size between points of 20 bp. Different positions having gaps were left out from the comparison. 100 Bootstraps were considered primarily. Kimura-2-parameter of T/t=1.5, were used to calculate the DNA distance and branch length was calculated by Neighbour-joining method. The colours are constant with the similarity curves.

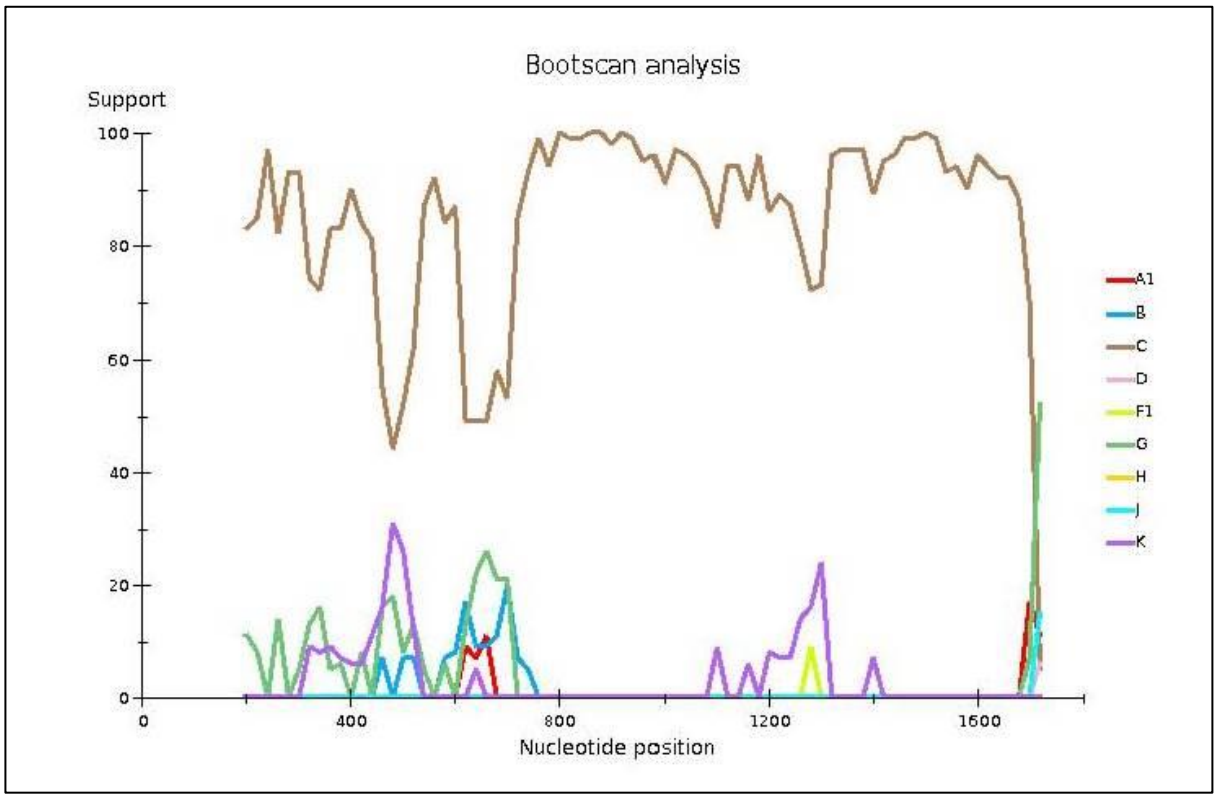

Figure 2.4: Plot of bootscan analysis generated by SimPlot.

Human immunodeficiency virus 1 msaha01612 pol gene for pol protein, partial cds
GenBank: LC570898.1

GenBank Graphics

>LC570898.1 Human immunodeficiency virus 1 msaha01612 pol gene for pol protein, partial cds

CCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAGAATAGGGGGCCAGATAAAAGAGGCTCTCT
TAGAYACAGGAGCAGATGATACAGTATTAGAAGAAATAAATTTGCCAGGAAAATGGAAACCAAAAATGAT
AGGAGGAATTGGAGGRTTTATCAAAGTAAGACAATATGATCAAATACCTATAGAAATTTGTGGAAAAAAG
GCTATAGGTACAGTATTAGTGGGACCCACACCTGTCAACATAATTGGAAGAAATATGTTGACTCAGCTTG
GATGTACACTAAATTTTCCAATTAGTCCCATTGAAACTGTACCAGTAAAATTAAAGCCAGGAATGGATGG
CCCAAAGGTTAAACAATGGCCATTGACAGAAGARAAAATAAAAGCATTAACAGMAATTTGTGAKGARATG
GAGAAGGAAGGAAAAATTACAAAAATTGGGCCTGAAAATCCATATAACACTCCAATATTTGCYATAAAAA
AGAAGGACAGTACTAAGTGGAGAAAATTAGTAGATTTCAGGGAACTCAATAAAAGAACTCAAGATTTTTG
GGAAGTCCAATTAGGAATACCACACCCAGCAGGGTTAAAAAAGAAAAAATCAGTGACAGTACTGGATGTG
GGGGATGCATATTTTTCAGTTCCTTTAGATGAAGRCTTCAGGAAATATACTGCATTCACCATACCTAGTA
CAAACAATGAAACACCAGGGATTAGGTATCAATATAATGTGCTTCCACAGGGATGGAAAGGATCACCAGC
AATATTTCAGARTAGCATGACAARAATCTTAGAGCCCTTTAGRGCACAAAATCCAGAAATAGTCATCTAT
CAATATATGGATGACTTGTATGTAGGATCTGACTTAGARATAGGGCAACATAGAGCAAARATAGAGGAAT
TAAGAGAACATCTATTAAGGTGGGGATTTACCACACCAGACAAGAAACATCAGAAAGAACCCCCATTTCT
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TGGACTGTCAATGATATACAAAAGTTAGTGGGAAAATTAAACTGGGCAAGTCAAATTTACCCAGGGATTA
AAGTAARGCAACTTTGTAGACTTCTTAGGGGGRCCAAAGCACTAACAGACATARTACCACTAACTGAAGA
AGCAGAATTAGAATTGGCAGAGAACAGGGAAATTCTAAARGAACCAGTACATGGAGTATATTATGACCCA
TCAAAAGACTTGATAGCTGAAATACAGAAACAGGGGCAGG

Human immunodeficiency virus 1 msaha001459 pol gene for reverse transcriptase, complete cds

GenBank: LC529744.1

GenBank Graphics

>LC529744.1 Human immunodeficiency virus 1 msaha001459 pol gene for reverse transcriptase, complete cds

ATGCGGATCACTCTTTGGCAGCGACCCCTTGTCGCAATAAAAATAGGGGGCCAAATAAAGGAGGCTCTCT
TAGACACAGGGGCAGATGATACAGTATTAGAAGAAATAAGTTTGCCAGGAAAATGGAAACCAAAAATGAT
AGGAGGAATTGGAGGTTTTATTAAAGTAAGACAATATGATCAAATAGCTATAGAAATTTGTGGAAAAAAG
GCTATCGGTACAGTATTAGTAGGACCCACACCTGTCAACATAATTGGAAGAAATATGTTGACTCAGCTTG
GATGCACACTAAATTTTCCAATTAGTCCTATTGAAACTGTACCAGTAAAATTAAAGCCAGGAATGGATGG
CCCAAAGGTTAAACAATGGCCATTGACAGAAGAGAAAATAAAAGCATTAACAGCAATTTGTGATGAAATG
GAAAAGGAAGGAAAAATTACAAAAATTGGGCCTGAAAACCCATATAATACTCCAATATTTGCCATAAAAA
AGAAGGACAGTTTTAAGTGGAGAAAATTAGTTCAAGATTTTTGGGAAGTTCAATTAGGAATACCACATCC
AGCAGGGTTAAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGGGATGCATATTTCTCAGTTCCTTTA
GATAAAGACTTCAGGAAATATACTGCATTCACCATACCTACTGTCAATGATATAAGTGTAAACAATGMAA
CACCAGGAATTAGATATCAATATAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAGAG
TAGCATGACAAAAATCTTAGAGCCTTTTAGGACACAAAATCCAGACATAGTCATCTATCAGTATATGGAT
GACTTGTATGTAGGATCTGACTATGAAATAGGGCAACATAGAACAAAAATAGAGGAGTTAAGAGAACATC
TATTAAGGTGGGGATTTACCACACCAGATAAGAAACATCAGAAAGAACCCCCATTTCTTTGGATGGGATA
TGAACTCCATCCTGAATACAGAAACAAATGGACAAGCTGAGACTTCAGGGAACTCAACAAAAGAACCCAG
AAAAGGATAGCTGGCAGAAGTTAGTGGGAAAATTAAACTGGGCAAGTCAGATTTACCCAGGAGTTAAAGT
RAAGCAACTTTGTAAACTTCTTAGGGGGACCAAAGCACTAACAGACATAGTACCACTAACTGAAGAAGCA
GAATTAGAATTGGCAGAGTACAGCCTATACGAACAGRGAAATTCTGAAAGAATCAGTACATGGAGTATAT
TATGACCCATCAAAAGACTTAATAGCTGAACAGGGGCATGACAGAACCCAGAA

Discussion

Present cross-sectional study conducted in Kolkata showing the presence of DRMs among people with pre-treatment drug resistance to HIV conferring resistance to PI is of concern. Present study provides one of the pioneer accounts of the prevalence of resistance to people with pre-treatment drug resistance to HIV representing a moderately diverse urban population in Kolkata. The results indicate that prevalence of mutations conferring antiretroviral resistance in people with pre-treatment drug resistance to HIV is low to moderate. The population in this study was in their initial stages of HIV disease and the resistance mutations screened for in this study are the ones listed by NACO, India. Subtype analysis of all the isolates found as subtype C, the predominant subtype in India. Before the current work, HIVDR mutations have not been reported by most of Indian studies [Eshleman SH.et.al.2005, Balakrishnan P.et.a;.2005, Sen S.et.al.2007, Soundararajan L.et.al.2007, Sen S.et.al. 2007] except a work carried out in Mumbai, on NRTI associated HIVDR mutations with the prevalence of 1.5 percent in PPDRH [Eshleman SH.et.al.2005, Balakrishnan P.et.a;.2005]. During recent days in northern India, A group of workers report the presence of DR mutations in 4.08 percent of people. Several studies from different regions of India carried out on people in ART have revealed a higher level of resistance in HIV-1 strain [Sen S.et.al.2007, Soundararajan L.et.al.2007, Sen S.et.al. 2007, Novak RM.et.al.2005]. Comparing with the frequency of initial resistance in different countries, recent studies in the United States and Europe have indicated that 10–20% of individuals are freshly come across with HIV and then infected by a drug-resistant HIV [Novak RM.et.al.2005, Cane P.et.al.2005] while studies from Japan and Korea have revealed prevalence rates are less than 10% [Ibe S.et.al.2008, Hwan Bang JI.et.al.2008]. Present study reveals the issue of the ARV drug resistance mutations among people with pre-treatment drug resistance to HIV (PPDRH) from Kolkata, which may pose challenges to first line ART program in India. The prevalence of initial HIV antiretroviral resistance in a diverse group of people who are attending ICTC

for first time will be the next big challenge for ART programme in this region [Emlet CA.et.al.2006]. Partial pol sequence (1310 and 1289 bases) of the strain niced_0025 and niced_0039 was recognized as possible mosaic sequence, which have highest homology with subtype C. Simplot study suggested that the sequences are may be putative C recombinants. Subtype C of HIV-1 is prevalent in India but several other subtypes and CFR of HIV-1 have been predominant in India [Gatell, J.M.et.al.2014]. Present study reveals the existence of ARV drug resistance on clinical outcomes. Clinical management for PPDRH in India will need to address the on growing problem of PDR. It is believed that transmitted drug-resistance mutations may persist for months, those mutations in the absence of selection pressure applied by drugs, revert to a more replication-competent variant. Such reversion makes the transmitted mutations undetectable. Thus, the rates of transmitted resistance observed in this study may be lower than the true counts that could be determined by resistance testing which could be done at the beginning of viral attack [Paranjpe, S.et.al.2016]. ARV drug mutation in HIV-1 is the key snags against the efficient HIV treatment [Little SJ.et.al.2002]. In India, rapid spread of HIV-1 make the country entered into the third stage of the epidemic. An increase in the use of ARV drug is observed as the reduction in cost of generic drugs in India. Analysis of DRMs at different locations for drug resistance in protease and RT gene sequences from HIV-1C (subtype) has been done in the present study [Pieniazek D.et.al.2000, Cornelissen M.et.al.1997, Becker-Pergola G.et.al.2000]. The implication of mutations on identified drug resistance locations and at locations recently revealed, as defined here, with relation to drug exposure in HIV-1 subtype C virus, is not clearly depicted in the previously published articles. Though, more accurate studies are required to determine whether the genetic backbone of HIV-1 subtype C provides different ways to find out to drug resistance, or can adjust the progression and effects of selected DRMs to HIV-1 subtype C may through some variations in the Pol enzyme [Becker-Pergola G.et.al.2000]. Present study, although limited to a total of 104 people with pre-

treatment drug resistance to HIV, offers some hope, that newly found subtype C viruses in different regions in southern Asia are not generally show higher expressions of drug resistance. Analysis of HIV-1 subtype C isolates from PPDRH may find out the clinical outcome of ARV therapy between naturally occurring variability derived drug resistance and treatment-related drug resistance [Boden D.et.al.1999]. The increasing rates of PPDRH observed in this study, recommend that resistance tests must performed periodically for pre-treatment drug resistance to HIV. Plasma HIV RNA of These people, who had attained pre-treatment drug-resistance, was not suppressed as voluntarily by potent antiretroviral therapy. The no to delayed response to ART including additional inadequate viral suppression allows further rounds replication of HIV genome, in presence of ART, which might, allows the selection of HIV-1 subtype C strain with added drug resistance. The existence of less options for primary treatment and suboptimal responses to treatment among the people with pre-treatment drug resistance to HIV, may seriously hinders the expected outcome as the decrease in disease progression and increase secondary transmission of drug-resistant HIV strain. The treatment strategies for people with pre-treatment drug resistance to HIV, throughout the world, should consider prevalence of drug resistance prior to the treatment [UNAIDS Report 2011].

Conclusion

Through the present study we show the pattern of HIVDR in the people with pre-treatment drug resistance to HIV in Kolkata. A high variation of major and accessary PI mutations, NRTI and NNRTI-resistant mutation was observed in ART-naive PHLV in Kolkata. Present outcome reveals that monitoring of drug resistance mutations along with DRM viruses prior to initiation of ART may deliver a logical basis for extensive inhibition and regulatory program of HIV.

Chapter 3

HIV-1 Diversity among HIV infected Individual under ART

Preamble

The estimation of adult (15-49 years age) HIV prevalence in India was 0.26% in 2015 with the total 2.11 million (1.71 – 2.64 million) HIV infected individual. Successfully implementation of National AIDS Control Program (NACP) by National AIDS ontrol Organization (NACO), it has been found that the new cases of HIV infection is declining phase in compared to 2002 to 2007. NACP has reached 66% to 32% decline in new HIV infection during five years [WHO, UNAIDS, UNICEF, Global HIV/AIDS response, 2011]. On April 1st, 2004, free antiretroviral Therapy has been initiated for the people living with HIV/AIDS and this program became very beneficial for the country as India harbors second largest number of PLHIV [Bhaskaran K. et.al.2008] or monitoring the treatment outcome, HIV drug resistance surveillance studies is necessary as the spread of HIV drug resistance is evidenced [Jahn A.et.al.2008]. The first line ART started in India with two nucleoside / nucleotide analogue reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) (Ives NJ.et.al 2001). The antiretroviral medication; zidovudine (AZT), abacavir (ABC), lamivudine (3TC), stavudine (d4T), didanosine (DDI) and emtricitabine (FTC) are available option for NRTI whereas only tenofovir (TDF) is available as nucleotide reverse transcriptase inhibitor. The efavirenz (EFV) and nevirapine (NVP) are the option for NNRTI. Viral load (VL) monitoring plays an important part of standard treatment protocol as it is selected for confirmation of treatment failure [Mills EJ.et.al.2011]. It is evidenced that the thymidine analogue a mutation (TAMs) is frequently observed for the patients without monitoring of viral load [UNAIDS progress report 2012]. As a result of cART administration, the morbidity and mortality of HIV infection is reduced robustly by suppressing HIV replication. The development of HIV drug resistance plays as barrier for the success of cART program. Routine primary drug resistance testing should be standard practice for the subjects those are initiating therapy. But a resource limited settings

like India, development of DRMs plays a negative impact in the prevention and therapeutic strategies. There is no representative data on primary DRMs after initiation of ART in India. A few data available from north-eastern part of India, regarding HIV DRMs are: M184V and L74V followed by the thymidine analog mutations (TAMs), M41L, K219E, D67N and T215S, for Nucleoside Reverse Transcriptase Inhibitor (NRTI) resistance; Y181C, K103N, V106M, G190A, K101E and Y188C for Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) resistance; and M46I, I47A, V82I and L90M for Protease Inhibitor (PI) resistance [Mills EJ.et.al.2009; Lakshmi R.et.al. 2010]. It is essential to access the effectiveness of ART program; the estimation of virological suppression among subjects with first line regimen is required. To monitor the treatment outcome, the resistance surveillance studies is necessary to prevent HIV drug resistance (HIV DR).

In eastern India, very few data is available on first line ART. Therefore, the aim of the present study is to determine the pattern of HIV DRMs in a large number of cART treated patients in this region, which could have a bearing on the future planning for ART drug procurement and distribution by the government agencies.

Material & Methods

Study Population and setting

HIV infected patients those were visiting ART center Kolkata were screened for eligibility for this study. Two main inclusion criteria were followed for recruitment in the present study. Age of the patient should be >18 years (adult) and CD4 cell count should be <200/ μl were considered for entry. The present study was approved by Institutional Ethical Committee and all the participants have given written informed consent.

At initial phase, maintaining the inclusion criteria total 57 individual were enrolled in the study. All the enrolled patients were screened for every six months starting from January 2013 to December 2015. During the course of study 13 patients were discarded from the study because they did not follow up. Rest 44 enrolled participants received first line ART drug regimen during January 2013 to December 2015. Genotypic drug resistance testing was done for the patients with HIV viral load >1000 RNA copies/ml.

Data Collection

The detailed treatment history was taken from all the enrolled participants. Among the participants those were reported no prior exposures to antiretroviral drugs were considered ART naive.

Specimen collection and processing

Ten milliliters (ml) whole blood sample were collected from each individual recruited in this study. CD4+T cells estimation was done by using three ml of blood. Within six hours of collection, the remaining blood was centrifuged at 400g for 10 minutes for plasma separation. After centrifugation, the plasma was aliquoted in 1 ml vial and stored at -70 °C. For HIV-1 RNA quantification and HIV-1 genotyping testing, fresh aliquots of plasma was used. WHO and HIVResNet Laboratory working group resistance testing guidelines were followed for HIV-1 genotyping [Eshleman H.et.al. 2004].

Confirmation of HIV-1 Seropositivity

The sero-positivity status of recruited participants was confirmed by three sets of Enzyme Linked Immunosorbent Assays (ELISA) which is considered as most sensitive assay.

CD4 Estimation

CD4 cell count were estimated using (BD FACS CALIBUR, BD Bioscience, CA, USA) flow cytometer.

Here whole blood samples were used for CD4 estimation and the whole procedure was done as per manufacturer's instruction and the data analysis was done by BD Multiset Software.

Procedure in brief:

- 50µl whole blood and 20 µl FACS CALIBUR reagents were added to True count tube containing the reference beads.
- The tubes were vortexed
- The tubes were incubated in dark at ambient temperature for 15 minutes.
- 450 µl of 1:10 diluted lysing solution was used to lyse RBCs at ambient temperature for 15 minutes in dark.
- The stained samples were acquired on FACS CALIBUR and analyzed using automated MULTISET software by automated gating.

Viral Load Testing

In this chapter, the quantitation of HIV-1 RNA in human plasma has been done by AMPLICOR HIV-1 MONITOR test which is an in vitro nucleic acid amplification test. The standard specimen processing procedure is used to quantitate HIV-1 RNA over the range of 400 – 750,000 copies/ml.

The AMPLICOR HIV-1 MONITOR Test is based on five major process: Specimen preparation; reverse transcription of (RT) of target RNA to generate complimentary DNA (cDNA); PCR amplification of target cDNA using HIV-1 specific complimentary primers;

hybridization of the amplified products to oligonucleotide probes specific to the target (s); and detection of the probe bound amplified products by colorimetric determination.

Pre-Amplification:

Reagent Preparation

- 100µl of HIV-1 Mn^{2+} was added to the entire tube of HIV-1 MMX.
- For proper mixing, the tubes were inverted for 10-15 times.
- 50µl of working Master mix was pipetted out.
- MicroAmp tray was stored at 2-8°C until further process.
- Specimen and Control preparation
- 70% ethanol was prepared.
- For preparation of working lysis reagent, HIV-1 QS was vortexed for at least 10 seconds. For one batch (upto 12 specimen and controls), 100 µl HIV-1 QS added to one bottle of HIV-1 lysis and mixed.
- After thawing the plasma specimen, the tubes were spun.
- 600 µl of working lysis reagent was added to each tube.
- 200 µl of sample was added to appropriately labelled tube and vortexed.
- For negative, low and high positive control, 200 µl NHP was added to appropriate tubes and vortexed.
- 50 µl of HIV-1 (-) C, HIV-1 L (+) C and HIV-1H (+) C were added to particular tubes.
- 10 minutes incubation was done at room temperature.

- 800 µl of 100% isopropanol was added to each tube and vortexed.
- Centrifugation was done at room temperature at 12,500 x g for 15 minutes.
- The supernatants were removed gently and discarded.
- 1ml 70% ethanol was added to each tube at room temperature and vortexed.
- Centrifugation was done for 5 minutes at 12,500 x g.
- The supernatants were removed gently and 400 µl of HIV-1 DIL was added to each tube and vortexed to resuspend the extracted RNA.
- The amplification was done within 2 hours.
- 50 µl control and samples were pipetted out into the appropriate MicroAmp reaction tube for further processing.

Reverse Transcription and Amplification

The amplification was performed at GeneAmp PCR system thermal cycler as per table 3.1

Table 3.1: Thermo cycling profile for Amplification.

Program	**Temperature and Time**
HOLD	2 minutes at 50°C
HOLD	30 minutes at 60°C
CYCLE Program (4cycles)	10 sec at 95°C, 10 sec at 55°C, 10 sec at 72°C
CYCLE Program (26cycles)	10 sec at 90°C, 10 sec at 60°C, 10 sec at 72°C
HOLD	15 mins at 7210 sec at 95°C, 10 sec at 55°C, 10 sec at 72°C

After removing the tray from thermal cycler, 100µl MONITOR DN was pipetted out in each reaction tube.

Detection

- 10X wash solution were prepared.
- 100 µl MONITOR MWP was added to each well (as per manufacturer's instruction). Rows A through F of the HIV-1 MONITOR MWP are coated with the HIV specific oligonucleotide probe; rows G and H are coated with the QS specific oligonucleotide probe.
- 25 µl of denatured amplicon was added to the HIV wells in row A of the MWP and was mixed up and down 10 times. Serial 5-fold dilution was made in HIV wells in rows B through F. Row F was mixed as before and 25 µl pipette tips were discarded.
- 25 µl of denatured amplicon to the QS wells in row G of the MWP and mixed. 25µl from row G to row H was transferred. 25 µl from row H was discarded.
- MWP lid was covered and incubated for 1 hour at 37ºC ±2 ºC.
- Washing was done as per automated washer program.
- 100 µl AV-HRP conjugate was added to each wells. Incubation was done at 37ºC ±2 ºC for 15 minutes.
- Working substrate solution was prepared by mixing SUB A and SUB B and 100 µl of working substrate solution was pipetted out into each well and it was kept at room temperature in dark.
- 100 µl stop solution was added into each wells. 450 nm wavelengths were used to measure the optical density.

Calculation of Results:

The calculation of HIV-1 RNA level was done as per manufacturer's instructions.

Determination of standard Test Results:

HIV-1 RNA copies/ml:

Total HIV-1 OD *Input HIV-1 QS copies/ PCR Reaction * 40 = HIV-1 RNA copies/ml

Total QS OD

Where:

Total HIV-1 OD = Calculated total HIV-1 OD

Total QS OD = Calculated total QS OD

Input HIV-1 QS copies / PCR = The number of copies of QS in reaction; this information is lot specific. Ref.: AMPLICOR HIV-1 Monitor test data card.

40 = factor to convert copies per PCR to copies per ml of plasma.

Viroseq™ HIV-1 Genotyping Assay:

HIV-1 genotyping assay was performed by using Viroseq™ HIV-1 Genotyping system with plasma. The procedure starts with isolation of RNA and high-speed centrifugation of plasma to pellet virus particles.

(i) Extraction of RNA

- Preparation of 70% ethanol was done and kept on ice.
- Viral lysis buffer was thawed and vortexed.
- After centrifugation, the residual supernatant was removed.
- 600µl of viral lysis buffer was added to each pallet.
- Vortexing was done for 3-5 seconds.

- Incubation was done at 15-25°C for 10 minutes.
- For precipitation of viral RNA, 600µl of 100% isopropanol was added to each tube.
- Voxtexing was done for 3-5 seconds and the centrifugation at 12,500-15,000 x g for 15 minutes was done.
- Without disturbing the pellets, the supernatants were removed by using fine tips.
- For washing the RNA pallet, 1 ml of freshly prepared 70% ethanol was added.
- After vortexing, centrifugation was done at 12,500-15,000 x g for 5 minutes.
- Residual supernatants were removed.
- Residual ethanol was removed carefully as it may inhibit reverse transcriptase reaction. The tubes caps were opened for at least 1 minute for air dry.
- The washed RNA pallet was resuspended with 50µl or 100 µl of RNA diluent if the viral load of the sample is known to be less than 15,000 cp/ ml or greater than 15,000 cp/ ml respectively. The RNA can be stored at -80° C for long term storage.

(ii) Reverse Transcription:

- Before starting the procedure, RT mix and Dithioretol (DTT) and RNA extract were thawed at room temperature, vortexed and spun.
- RT mix was prepared for required sample no as per table no 3.2
- The RT mix was vortexed gently for 1-2 second to mix and pulse spun at low speed.
- In 0.2ml reaction tube, 10 µl RNA extract, positive and negative controls were added. The tubes were placed in thermal cycler followed by thermo cycling profile as per table 3.3

- Program A and B was started and before end the program B, the thermal cycler was paused.
- After removing the sample from thermal cycler, 10 µl of RT mix was added to each reaction tube.
- The thermal cycler program was resumed and samples were returned back.

Table 3.2: RT Mix Reagents

Reagent	Volume (µl/ smaple)
HIV RT Mix	8
RNAse inhibitor	1
MuLV RT	1
100mm DTT	0.4
Final Volume	10.4

After completion of run, the samples were held at 4°C for at least 10 minutes.

Table 3.3: Thermo cycling program for Reverse Transcription

Program	Temperature	Time	Process
A	65ºC	30 seconds	Relax and denature RNA secondary structure
B	42 ºC	5 minutes	Cool to optimal enzyme activity temperature
Manually PAUSE the thermo cycler at this point and continue with the next step. 15 minutes time available to perform the test			
1	42 ºC	60 minutes	Reverse Transcription
2	99 ºC	5 minutes	Inactivate MuLv RT
3	4 ºC	hold	Holds until you are ready

Table 3.4: PCR mix reagents

Reagent	Volume for one sample (µl/ sample)
V2 PCR Mix	29.5
AmpliTaq Gold	0.5
UNG	1.0
Final Volume	31

Table 3.5: Thermo cycling Program for PCR

Cycle	Temperature	Time
--	50 ºC	10 minutes
--	93 ºC	12 minutes
40	93 ºC	20 Seconds
	64 ºC	45 Seconds
	66 ºC	3 minutes
--	72 ºC	10 minutes
--	4 ºC	Hold

(iii) PCR Amplification

- PCR brew mix was vortexed for 3-5 seconds, spun and placed on ice.
- 30 µl of PCR Mix was transferred to each RT reaction tube.
- Amplification was proceeded with thermo cycling profile describe in Table 3.5
- After completion of this process, the samples were preceded for purification.

(iv) Microcon Purification

- Microcon 100, microconcentrator used for the purification process that comes along with the viroseq kit.
- 1.5 ml microcon collection tubes were labeled properly.
- Microcon concentrator were assembles as per manufactures instructionand 300µl KCL buffer was pipetted onto the top of the microconcentrator.
- On the top of the microconcentrator, 50 µl of NA CR product was pipetted from thermal cycler.
- Centrifugation was done for 15 minutes at 450-550 g at room temperature.
- To the sample reservoir, 300 µl DEPC treated water was added and centrifugation was done for 15 minutes at 450-550 g at room temperature.
- Finally, to the sample reservoir, 35µl of DEPC treated water was added that was placed upside down on the top of the second labeled 1.5ml vial and the first vial was discarded.
- Centrifugation was done 15 minutes at 450-550 g at room temperature.
- The concentrated purified PCR product was transferred in 35µl DEPC treated water into the new vials.
- The tubes were spun at low speed at room temperature for 5 – 10 seconds to remove any drops of the side of the tube.
- The samples were analyzed on 1% agarose gel as per standard protocol.

(v) Cycle Sequencing PCR product and purification:

The diluted PCR products were sequenced thermo cycling profile was used as per the standard protocol.

For purification, an isopropanol (ACS Grade Reagent, Sigma-Aldrich, MO, USA) couple with centrifugation at 2000 x g for 45 minutes was used.

- Before starting the procedure, the purified PCR product was diluted to 30 ng/µl (15 – 25 µl total volume) using DEPC treated water.
- The sequencing PCR was done by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystem, CA, USA). The Big Dye chemistry was carried out with HIV-SEQ mix and diluted purified PCR product with final reaction volume 10 µl.

Table 3.6: Preparation of In-house HIV-SEQ Mix for each sequencing primer

Reagents	Volume
Big Dye Terminator Ready Reaction Mix	2µl
5x sequencing buffer	2µl
Primer (2pmoles/2µl)	2µl
DNase, RNase free water	2µl
HIV-SEQ mix was prepared for each sequencing primer	

- Diluted, purified PCR products and the sequencing primers were thawed.
- HIV Seq mixes was pulse vortexed.
- To the appropriate well of the plate, 8 µl HIV Seq mix were added followed by necessary precautions as per manufacturer's instruction.

- PCR product was vortexed. 2µl diluted, purified PCR product was added to the well into the plate.
- Centrifugation and spinning was done as per manufacturer's instruction
- Cover the plate with a rubber 96 well plate septa.
- Incubate sequencing Reactions in the thermal cycler using following program

Table 3.7: Thermo cycling profile for Sequencing PCR (Big Dye Chemistry)

Number of cycles	Temperature	Time
25	96 ºC	10 Second
	50 ºC	5 second
	60 ºC	4 minutes
--	4 ºC	hold

Table 3.8: Primer used for HIV-1 Sequencing PCR

Primer	Sequence
2135 F	5'- TTT AGAGCAGACCAGAGCCAACAG C -3'
2493 F	5'- CCT GTC AAC ATA ATT GGA AG -3'
3012 F	5'- GGA TCA CCA GCA ATA TTC -3'
2557 R	5'- GGT ACA GTT TCA ATG GGA C -3'
3117 R	5'- CCC TAT TTC TAA GTC AGA TCC -3'
3338 R	5'- TTT TCC CAC TAA CTT CTG TAT AGT CAT TG -3'
3403 F	5'- GGG CCA AAG TAC TAA CAG -3'
3768 F	5'- CAA GCC ACC TGG ATT CCT GAG TG -3'
3999 R	5'- CCT GAA TCT TGC AAA GCT AG -3'
4381 R	5'- CCT GGA CTA CAG TCT ACT TGT CCA T-3'

(vi) Post Sequencing PCR Precipitation:

- Using 200µl multichannel pipettor, 80µl of 80% isopropanol to each 10 µl sequencing reaction was added.
- The plate was covered immediately with septa
- Mixing was done by inverting the plate three times.
- The plate was incubated for 15minutes at room temperature in dark.
- Centrifugation was done for 45 minutes at 35R at 2000 x g
- After removing the cover, the plate was inverted on a folded tissue paper and centrifugation was done at 700 g x for 1 minute.
- The plate was dried for 5 minutes
- The samples were resuspended in 20µl of HiDi Formamide cover with septa.
- Finally, the plate was loaded into the sequencer for sequencing.

(vii) Analysis

- ABI PRISM 3100xl Genetic Analyzer and Viroseq software vs 2.8:
- The sequencing was carried out on automated (16 capillary) ABI PRISM 3100xl Genetic Analyzer using data collection software v3.0 and sequence analysis software v2.8 was used to assemble the chromatographs from the seven primers and generated a contiguous sequence spanning the entire protease gene, and upto codon 335 of the Reverse Transcriptase gene (RT). Points of variance were identified comparing the consensus sequence to a known reference strain, HXB-2.
- The viroseq software v2.8 was used to analyze ABI files for each sample.
- Mixtures were called as per the following criterion:

- Two opposite sense sequence segments containing a secondary peak clearly above the local noise.
- One sequence segment containing a secondary peak at 30% of the primary peak and 3X background noise. One segment containing a secondary peak at 30% of primary peak and the segment in the opposite direction confirms the mixture.

Quality Control

A negative, low-positive and high positive control samples were run with every batch for quality control of HIV-1 genotyping. The success in RT-PCR and HIV-1 genotyping assay were ensured by positive controls. For ensuring good sequence quality, the high positive control was sequenced before genotyping the IV-1 samples, precluding editing mistakes.

Statistical Analysis

All the data collected on a predesigned paper and simultaneously transferred to Microsoft Excel spread sheet. To minimize keyboard errors, all the entries were double checked at the entry level. Baseline clinical and biological characters of the study subjects were summarized as frequency (%) for the categorical variables; and mean ± standard deviation (SD) or median {Interquartile range (IQR)} for quantitative variables.

Clade typing

REGA HIV-1 sub-typing tool from the Stanford HIV drug-resistance database (http://hivdb.stanford.edu/) was used to define HIV-1 subtype .Worldwide subtype references were obtained from the Los Alamos HIV database.

Phylogenetic tree

Clade typing and phylogenetic reconstructions were performed using the REGA sub typing tool of the HIV drug resistance database. Clustal W multiple sequence alignment program was used to align nucleotide sequences. Phylogenetic analysis was conducted using MEGA v3.0 software. The neighbor-joining method and Kimura parameter model were used for tree construction with reliability estimated from 1000 boot stap replicates [Thompson JD.et.al.1994; Kumar S.et.al.2004]

Result

Genotyping results were obtained for samples from a total of 44 patients. Their important baseline characteristics are summarized in Table 3.9.

Table 3.9: Baseline Characteristics of the Study Participants

Parameters	Value (n=44)
Age(years)	
Mean±SD	41.4±8.56
Range	41.4±8.5
Sex	
Male/female	30(60%) /14(31.81%)
BMI, Kg/m^2	
mean ± SD	20.5±7.13
Range	14-31
Risk Factors	
Heterosexual	35(79.54%)
Blood Transfusion	5(11.36%)
Intravenous drug user	4(9.09%)
Educational status	
Literate	27(61.36%)
Illiterate	17(38.64%)
Locality	
Urban	15(34.09%)
Rural	29(65.91%)
Marital status	
Married	36(81.81%)
Unmarried	4(9.09%)
Widow	3(6.81%)
Widower	1(2.72%)

Parameters	Value (n=44)
Duration of ART,	
months, median (range)	34(11-109)
History of change of ART, n (%)	
No change	32(72.7%)
Once	8(18.1%)
Twice	4(9.09%)
Median CD4 cell count	
Cells/μL (IQR)	96(45-184)
Median Viral load	
log_{10} copies/mL (IQR)	5.10(3.37-7.02)
Starting regimen of ART, n (%):	
D4T+3TC+NVP	20 (45.45%)
ZDV+3TC+NVP	18 (40.9%)
D4T+3TC+EFV	4(9.09%)
ZDV+3TC+EFV	2 (4.54%)
Opportunistic infections, n (%):	
Cryptosporidiosis	1(2.275%)
Cytomegalovirus	3(6.81%)
Pulmonary TB	14(31.81%)
Extrapulmonary TB	9(20.45)
Pneumocystis jirovecii (carinii)	2(2.275%)
Pneumonia (PCP)	
Herpes Zoster	6(6.81%)

SD= Standard deviation; BMI= Body mass index; ART= Antiretroviral treatment; D4T= Stavudine; 3TC= Lamivudine; NVP= Nevirapine; ZDV= Zidovudine; EFV=Efavirenz; PI= Protease inhibitors; TB= Tuberculosis.

The mean age (±Standard Deviation [SD]) of participants, of which 30 were males and 14 females, was 41.4 (±8.56) years. Their median duration of ART use was 34 months (range: 11-109 months). The median CD4 count was 96 (range: 45-184) cells/mm^3, and median log_{10}viral load/ml was 5.10 (range: (3.37-7.02). ART failure was documented for all individuals by their respective ART clinics.

First line antiretroviral drugs used by the Indian national ART programme are zidovudine (ZDV), lamivudine (3TC), stavudine (D4T), nevirapine (NVP) and efavirenz (EFV). The most common ARV regimen patients were taking at the time of enrolment was ZDV+3TC+NVP (45.5%), followed by D4T+3TC+NVP (34.09%), D4T+3TC+EFV (9.09%) and ZDV+3TC+EFV (4.54%) respectively. Pulmonary tuberculosis was observed to be the most common opportunistic infection in 14 (31.81%) of the study participants, followed by Extrapulmonary TB in nine (20.45%), Herpes Zoster infection and cytomegalovirus was encountered in 3 (6.81%). PCP pneumonia and cryptosporidiosis was found in one (2.275%) person. A total of 44(100%) participants had mutations conferring resistance to at least one ARV drug (Table 3.9).

Table 3.10: Frequency of Major HIV NRTI, NNRI & PI Drug Resistance Mutations

Mutation	No. of Patients(%),n=88
NRTI DRMs:	34(77.27)
T69R	2(4.54)
K70A	2(4.54)
M184V	15(34.09)
L74V	1(2.27)
D67N	2(4.54)
M41L	5(11.36)
D67S	5(11.36)
T69N	5(11.36)
K70R	5(11.36)
T215F	11(25.0)
K219E	9(20.45)
V75L	1(2.27)
V75I	2(4.54)
T69G	3(6.81)
NNRTI DRMs:	15(34.09)
Y181C	2(4.54)
V189D	1(2.27)
Y188L	8(18.18)
K238T	1(2.27)
K101E	2(4.54)
K103N	3(6.81)
G190A	2(4.54)
A98G	3(6.81)
Y181G	1(2.27)
E138A	1(2.27)
PI DRMs:	7(15.90)
V82S	1(2.27)
V82L	3(6.81)
I84V	3(6.81)

HIV= Human immunodeficiency virus; DRM= Drug resistance mutation; NRTI=Nucleoside reverse transcriptase inhibitors; NNRTI= Nonnucleoside reverse transcriptase inhibitors; PI= Protease inhibitors.

NRTI mutations were seen in 34 (77.27%) patients and 15 (34.09%) had NNRTI mutations. PI mutations were seen in five (11.3%) individuals. The most common NRTI mutation observed were M184V (34.09%), T215F (25.0%) and K219E (20.45%), and Y188L (18.18%), K103N (6.81%) and A98G (6.81%) among NNRTI associated ones. PI DRMs were observed in 7/44 (15.9%) patients, with V82L, V82S and I84V being the commonest. Only one class resistance was seen in four (9.09%) individuals, of which two (2.3%) were with an NRTI and one each had an NNRTI and a PI resistance (2.27%).

Two class resistances were seen in 37 (79.7%) persons with combinations of NRTI+NNRTI, NRTI+PI and NNRTI+PI. A total of 30 (68.18) patients fell in the category of NRTI + NNRTI combination followed by 4 (9.09%) and 3 (6.81%) patients respectively for NRTI + PI and NNRTI + PI patients. Three (6.81%) patients out of a total of 44 had resistance to all three classes of ARV (Table 3.10).

Table 3.11: Frequency of Drug Resistance Mutations by drug class

Mutation	**No. of Patients(%),n=44**
No mutation	Nil
One class mutations	4(9.09)
Only NRTI	2(4.54)
Only NNRTI	1(2.27)
Only PI	1(2.27)
Two class mutations	37(84.09)
NRTI + NNRTI	30(68.18)
NRTI + PI	4(9.09)
NNRTI + PI	3(6.81)
Three class mutations	3(6.81)

NRTI= Nucleoside reverse transcriptase inhibitors; NNRTI= Nonnucleoside reverse transcriptase inhibitors; PI= Protease inhibitors.

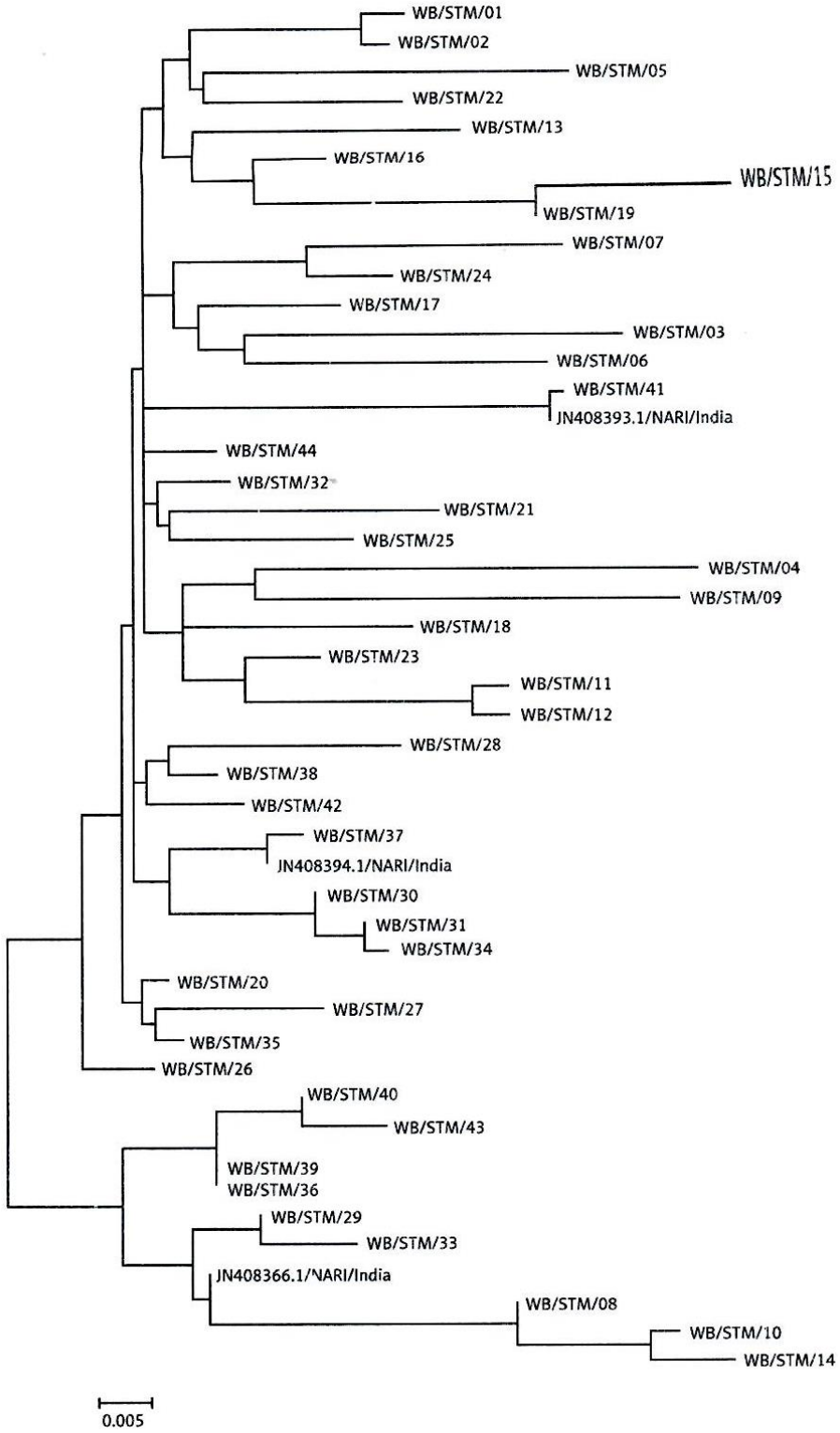

Figure 3.1. Molecular phylogenetic analysis by maximum likelihood method: The evolutionary history was inferred by using the Max- imum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (–5348.7146) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and

BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 47 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and miss- ing data were eliminated. There were a total of 887 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Discussion

Earlier studies on HIV drug resistance from western countries were based on HIV-1 subtype B. So, the studies on HIV non-B subtypes and its associated literature were rare. HIV-1 subtype C has been found in all infected individual in this study. Clusters of all isolates were present around the Indian HIV-1 reference strains JN408366.1, JN408394.1 and JN408393.1 from NARI (National Aids Research Institute), Pune (Fig. 3.1). This study establishes that the HIV-1 subtype C is predominant circulating strain in West Bengal as we can see in other parts of the country [Kantor R.et.al.2004; Khan IF.et.al.2007; Gadkari DA.et.al 1998]. Consequently, some of the drug resistance characteristics of mutations identified in this study reproduce those seen elsewhere with HIV-1 subtype C. In several studies, it is evidenced that M184V and Y188L emerge as the most prevalent NRTI and NNRTI mutations respectively with HIV-1 C subtype. This study is one of the few reports from resource-limited settings describing operational feasibility and programmatic challenges in relation to management of patients living with HIV who have suspected first-line ART failure.

A cocktail of three antiretroviral drugs is used as a combinatorial antiretroviral therapy (cART) that can effectively suppress viral replication, reduce rates of transmission, and improve patients' life expectancy by prolonging the onset of AIDS [Deeks SG.et.al.2011].

Nevertheless, cART does not fully restore health, as patients on cART still experience chronic inflammation and increased rates of non-AIDS morbidity and mortality [Buzon MJ.et.al.2011]. Moreover, current cART regimens are unable to cure HIV-1 patients, as these drugs cannot eradicate latent viral reservoirs and may also fall short in completely suppressing viral replication despite drug intensification [Wong JK.et.al.1997; Finzi D.et.al.1997]. Thus, nearly all HIV-infected individuals on cART will need to maintain their medications for the entirety of their lives, resulting in considerable expense, development of resistance, and toxic side effects. Because of the limitations and adverse effects associated with current cART regimens, it is necessary to develop alternative therapeutic strategies that are safer, more efficacious, and more resistant to viral escape. Such emerging therapeutic strategies include gene-based and nucleic-acid-based therapies that are based on gene editing, ribozymes, and RNA interference (RNAi). Recently diagnosed individual from Kakinada, Mumbai and Northern India and previous drug resistance surveillance studies showed <5% DRM prevalence (. Burnett JC.et.al.2012). Earlier studies from different parts of India indicate that the overall prevalence of primary DRMs is moderate (5-15%) [Yukl SA.et.al.2010]. The overall prevalence of RT DRM for Indian strains from treatment –naiive patients was determined to be 2.5%, and most of our samples and sequences originated from highly HIV- prevalent states of India. There has been a gradual increase in DRM in therapy-naiive patients over the past decade, but the overall prevalence has remained below the WHO threshold level of drug resistance (<5%). Our study also showed that there has been a significant rise in NRTI mutations and not in NNRTI mutations.

Conclusion

More recently, ART scaling-up in resource-limited settings is resulting in the occurrence of primary mutations in developing countries, as well. The world health organization (WHO) recommends periodic surveillance of transmitted drug resistance (TDR) mutations in drug-

naïve, recently infected individuals in distinct geographical areas. Furthermore, current treatment guidelines recommend routine laboratory testing to assess drug resistance-associated mutations (DRAMs) in patients with acute and chronic infections prior to ART initiation to optimize the treatment regimen. These are particularly recommended in countries scaling up ART and in areas where primary resistance has been consistently documented. Lack of testing for baseline resistance, in addition to, other factors including interruption in treatment due to disruption in drug supply, or as a result of financial restrictions and improper administration of drug regimens are the major causes for the occurrence and expansion of drug resistance in developing countries.

Chapter 4

HIV-1 Diversity among AIDS patients under ART presenting virological failure

Preamble

HIV is managed by Anti-retro viral therapy (ART) successfully but it leads to HIV DR Mutations [Smith DM.et.al.2008; Balakrishnan P.et.al.2005]. Development of HIVDR is common among people living with HIV (PLHIV) those fails in ART [Vidya M.et.al.2009; Hira SK.et.al.2004]. According to WHO, ART failure is detected early by Viral Load estimation that to identify virologic failure. World Health Organization (WHO) strongly recommends Viral Testing for all eligible subjects registered for anti-retroviral therapy (ART) for early detection of ART failure. But as the viral road testing requires resource rich settings, most of the country does not have the facility. As a result increasing of HIV drug resistance among treatment experienced patients are common. In India the second line ART regimen constitute zidovudine (ZDV), lamivudine (3TC), tenofovir (TDF), and boosted lopinavir/ ritonavir (LPV/r); familiarized in controlled phase. The patients on first line ART shows failure in clinical and/ or immunological and/ or virologic parameters for six months or more are eligible for shifting from first line ART to second line ART. PLHIV fails in second line ART demands third line ART [Zhang F.et.al.2009].

The enzyme protease plays an important role for HIV replication and completion of its life cycle. So, Protease (PR) is used as an important target for ART and different protease inhibitor (PIs) are approved and commonly used. The second line ART comprise of single drug of PIs with two nucleoside reverse transcriptase inhibitors (NRTIs). That occurrence of viral variants resistance to PIs is evidenced when PI based ART fails [Me´decins Sans Frontie`res (MSF) Access campaign 2013, 16th edn, Geneva, Switzerland; Condra JH.et.al.1996; Hertogs K.et.al.2000; Miller V.et.al.2001; Kandathil AJ.et.al.2009; Gupta A.et.al.2010]. The replacement of amino acid alters the PR catalytic activity that consequently affects virus replication capacity marked as major mutation [Orrell C.et.al.2009; Walmsley S.et.al.2002; Chen Z.et.al.1995]

So, second line ART based on PI becomes extremely effective [Kozal M.et.al.2004]. Globally, introduction of generic first line ART significantly reduced morbidity and mortality. Absence of viral load testing, virologic failure becomes undetected at early stage unless the patients develops clinical or immunologic failure and as a result patient continues on failing regimen and buildup of drug resistance mutations [Desai M.et.al.2012].

In India, HIV DR to first line ART is reported widely as a result the PI based second line ART is upgraded [Desai M.et.al.2012; Radhakrishnan P.et.al.2007; List of countries by HIV/AIDS prevalence rate 2012;] The outcome of second line ART remains blurred without drug resistance assay and regular virologic failure monitoring. Therefore, beside clinical, virological and immunological parameters, assessment of emergence of drug resistance mutations is important for better understanding the treatment outcome of second line ART.

From eastern India, this study aimed to investigate the extent IV drug resistance mutations among virologic failure people living with HIV (PLHIV) on second line ART in Kolkata. .

Materials and Methods

Study Population and setting

The study was conducted at ICMR-national Institute of Cholera and Enteric Diseases, Kolkata with the approval of ethical committee. The subject those are in 2nd line ART has been recruited approximately five years duration. A total of 365 HIV/AIDS patients has been recruited following Helsinki declaration 1975 and revision in 2000. The following inclusion and exclusion criteria have been set for recruitment of patients in the study.

Inclusion criteria:

a) HIV/AIDS patients those are in 2nd line ART and consenting for this study.

b) Age >18 years

c) HIV-1 viral load (HIV-1 RNA level) more than 1000 copies/ml (virologic failure)

Exclusion criteria:

a) Insufficient sample volume drawn from the patient.

b) Patients those were missing in follow up.

c) Co-infected patients with HIV-2 and/ or HIV 1/2.

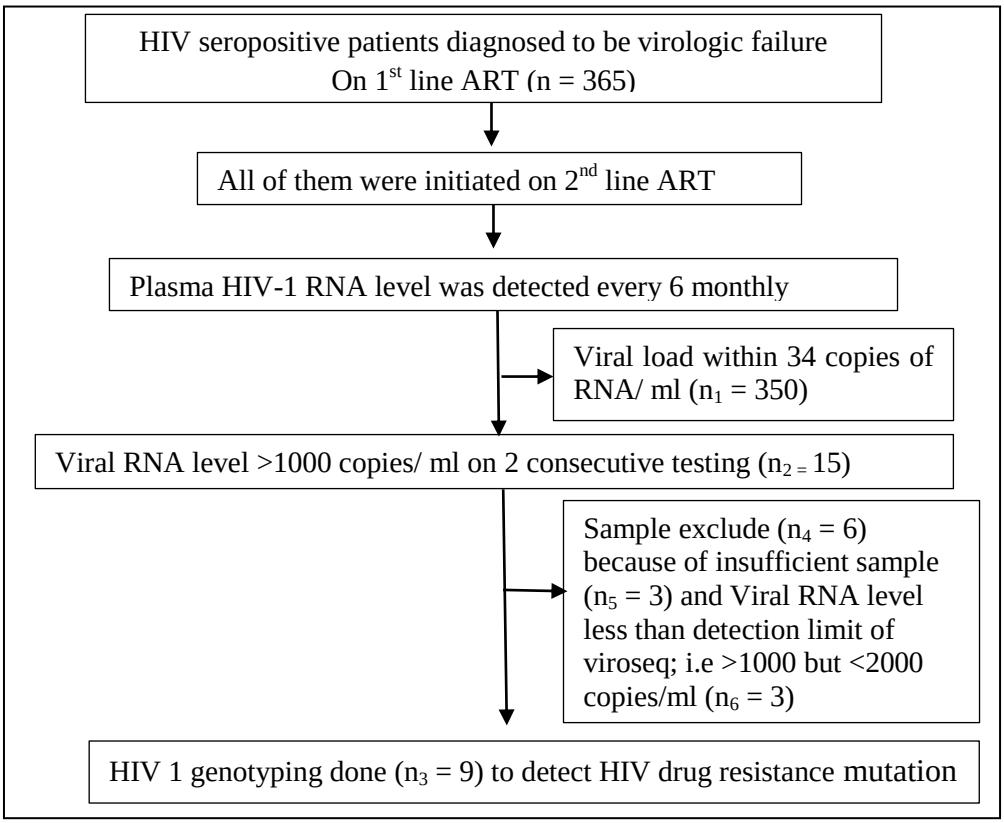

Figure 4.1: Flow diagram for recruitment of study population

Specimen collection and processing

A total of 10ml blood was collected from each recruited patients in the study. Among this 10 ml blood, 3 ml was used for CD4 T cell count estimation. After that the centrifugation was done for separation of plasma. The sample was aliquoted in 1 ml vial and stored at -70ºC for further processing.

Viral Load Estimation

AMPLICOR HIV-1 Moitor test, version 1.5 (Roche Molecular Systems Inc., Branchburg, NJ, USA) was used for HIV-1 viral Load estimation.

There are several methods for quantitation of HIV-1 virus level in peripheral blood. Here nucleic acid amplification technology was done for measurement of viral RNA in plasma.

Five major steps are involved to complete total procedure in AMPLICOR HIV-1 Moitor test. These steps are: i) specimen preparation, ii) Reverse transcription (RT) of target RNA to generate complimentary (cDNA), iii) PCR amplification of target cDNA using HIV-1 specific complimentary primers, iv) hybridization of the amplified products to oligonucleotide probes specific to the targets and v)detection of probe bound amplified products by colorimetric detection.

The details procedure has been described in previous chapter (Chapter-3; Section: Material and Methods).

CD4 estimation

Flow cytometer (BD FACS CALIBUR, BD Biosciences, CA, USA) was used to estimate CD4/CD8+ T cell.

BD FACS Caliber uses flow cytometer to determine percentage and absolute count of CD4 cells. The details procedure has been described in previous chapter (Chapter-3; Section: Material and Methods).

HIV-1 Genotyping

Viroseq HIV-1 Genotyping Systems (Abbott diagnostics, Wiesbaden, Germany) was used for permong HIV-1 Genotyping.1.8 kb of protease – RT region of HIV-1 pol gene were

sequenced as per manufacturer's instruction. The details procedure is described in (Chapter-3; Section: Material and Methods).

Quality Control

In every batch, high positive, low positive and negative control samples were run and it has been discussed in (Chapter-3; Section: Material and Methods).

Clade typing

REGA HIV-1 subtyping tool as discussed in Chapter-3; Section: Material and Methods has been used for clade typing.

Statistical Analysis

Data analysis was done by employing Microsoft Excel using Annova for clinical and biological characters of patients to find out frequency (%) for categorical statistical parameter.

Results

The viral loads of samples (n=9) genotyped were within the limit of 2990-4.49×10^6 RNA copies/ml. All HIV-1 samples were detected as subtype C (Table 4.1). Median age of the study group was 36 years (range: 30-46 years). The median CD4 count was 110 cells/μL (range: 51-475 cells/μL), and the median plasma HIV RNA load was 41800 copies/mL (4.60 log10), range: 2990(3.47 log10) – 4490000 (6.65 log10). HIV DRMs were detected from 5 patients, while from other 4 patients no major or minor HIVDR was detected. The Drug Resistance result showed that (for NRTI based drugs) resistance to drugs 3TC and FTC was among 80% of the patients. AZT, D4T and ABC resistance was seen in 40% of the patients. DDL resistance was seen in 20% of the patients. M184V that imparts resistance to NRTIs,

lamivudine, and emtricitabine, was seen among 80% of the test individuals. M41L mutation was also prevalent in 80% of the cases.

NRTI mutations T69D, T215Y, D67N were seen in 2 individuals whereas accessory NRTI mutations T215F and L74I were seen in one patient each. For NNRTI based drugs, NNRTI mutation, A98G and Y181C was predominant with 80% individuals, conferring resistance to DLV and NVP followed by K101E and G190A(40%), conferring resistance to EFV and ETR. Patients having NNRTI mutations were mostly due to the limited impact of NNRTI resistance mutations on viral fitness. A98G and Y181C were the predominant NNRTI mutations observed and these have little or no effect on replication capacity. Accessory NNRTI mutations K103N, E138K and V108I were seen in one patient each (Figure 2). PI mutations, l54V, A71V, V82A and M46L were seen in 40% of the individuals conferring resistance to drugs IDV, SQV, LPV, NFV and ATV. Accessory minor PI mutations L10V, L24I, M46I, I50L, K43T, F53L, T74P, L90M and L10I were seen in one patient each (Figure 3). Among the secondary PR mutations, amino acid variants at 7 polymorphic positions (codons 10, 20, 36, 63, 71, 77, and 93) were prevalent.

These mutations do not cause drug resistance by themselves but increase drug resistance when present together with other mutations thereby compensating the decrease in catalytic efficiency caused by other PR mutations. I54V, A71V, V82A and M46Lwere predominantly observed among participants in this study. Out of the 9 patients studied for 2nd line ART failure, in 4 PLHIVs no mutation was found with reference to Stanford HIV drug-resistance database (http://hivdb.stanford.edu/) for such sequences. One of the individuals showed only NNRTI mutations. A98G, K103N, Y181C conferring resistance to DLV, NVP and EFV (Table 4.2: for drug name abbreviations).

Table 4.1: Variables of PLHIV (n=9) failing on second-line ART genotyped

Variables	Summary
Age (yrs), median (IQR)	36 (30 -46)
Male	89%
Female	11%
Median C4 cell count, cells / µl (IQR)	110 (51 - 475)
Median Viral load, log10 copies/ml (IQR)	4.60 (3.47 – 6.65)
Viral load, log10 copies/ml	
<4.0	4 (44.4%)
4.0-4.9	3 (33.3%)
≥5.0	2 (22.2%)
HIV-1 subtypes, (%), Subtype C	100%

Table 4.2: Drug name abbreviations

Abbreviations	Full Name
3TC	Lamivudine
ABC	Abacavir
APV	Amprenavir
ATV	Atazanavir
ATV/c	Atazanavir/ cobicistat
ATV/r	Atazanavir/ ritonavir
d4T	Stavudine
ddl	Didanosine

DLV	Delavirdine
DRV	Darunavir
DRV/c	Darunavir / cobicistat
DRV/r	Darunavir /ritonavir
EFV	Efavirenz
EFV/c/TDF/FTC	Efavirenz/ cobicistat/ tenofovir disoproxilfumerate/ emtricitabine
ETR	Etravirine
`FTC	Emtricitabine
IDV	Indinavir
LPV	Lopinavir
LPV/r	Lopinavir/ritonavir
MVC	Maraviroc
NFV	Nelfinavir
NVP	Nevirapine
RAL	Raltegravir
RPV	Rilpivirine
RTV	Ritonavir
SQV	Saquinavir
SQV/r	Saquinavir/ritonavir
TDF	Tenofovir disoproxil fumarate
TPV	Tipranavir
TPV/r	Tipranavir/ritonavir

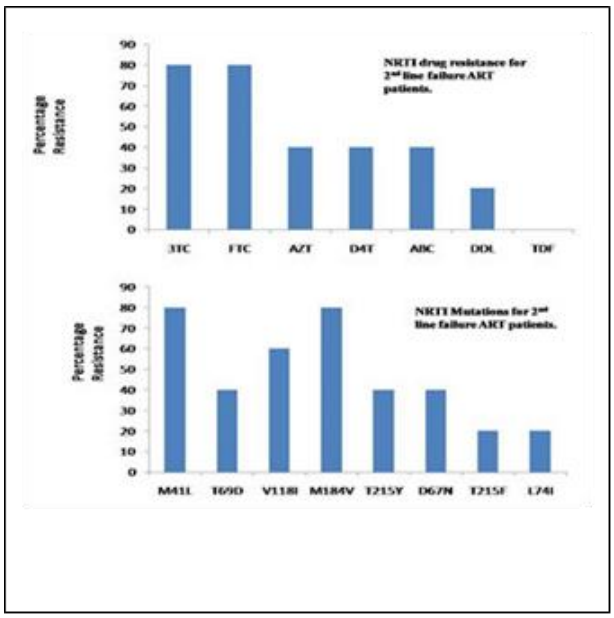

Figure 4.2: NRTI drug resistance and mutations for 2nd line failure ART patients.

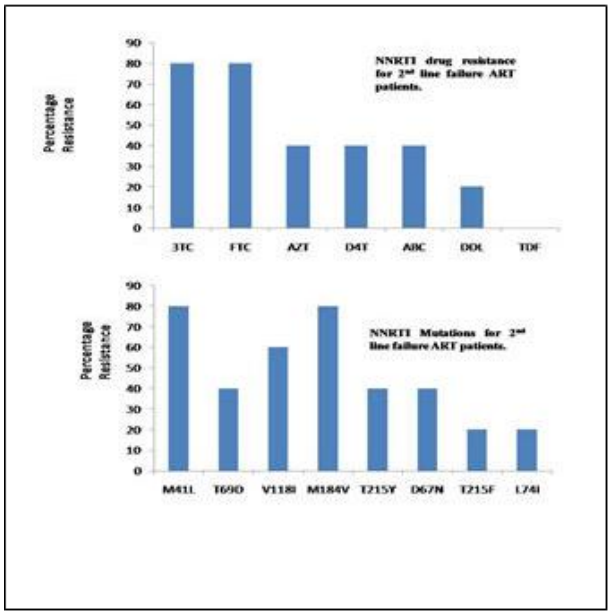

Figure 4.3: NNRTI drug resistance and For 2nd line failure ART patients.

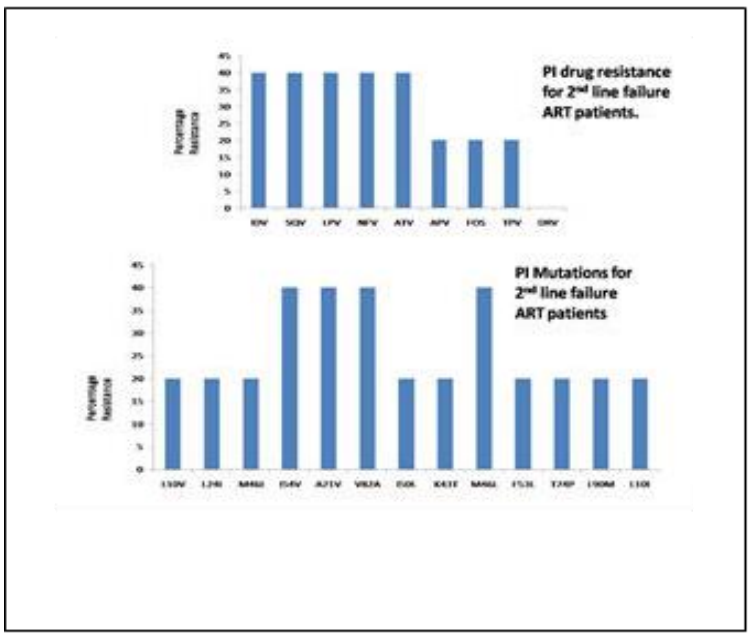

Figure 4.4: PI drug resistance and mutations for 2nd line failure ART patients.

Discussion

Studies on HIV drug resistance mutations among second line ART failure patients from eastern India reveals mutations among NRTI based drugs predominantly for M184V and M41L, and for NNRTI based drugs, A98G and Y181C with 80% conferring resistance to DLV and NVP. For PI mutations, l54V, A71V, V82A and M46L were seen in 40% of the individuals conferring resistance to drugs IDV, SQV, LPV, NFV and ATV. However, the most interesting observation was absence of HIV DR mutation in 4 patients out of 9 patients on 2nd line ART failure. Pattern of mutations suggests that majority of the patients participated in this study remain susceptible to DRV/r, followed by TPV/r. Of the 2 pathways that contribute to LPV resistance the IDV-like pathway caused by mutations at positions l54V, A71V, V82A and M46L was frequently observed among the ATV- and IDV-exposed groups. This could be because patients in this study had been increasingly substituted from IDV- to ATV-based regimens. Randomized clinical trials in developed countries show that the combination of raltegravir, ETR, and DRV/r was well tolerated and was associated with the rate of virologic suppression similar to that expected in treatment-naive patient [Yazdanpanah Y et. al. 2009].

This is highly contrasting compared to 100% HIV DR mutilations observed among patients on first line ART failure from the same geographical region [Dutta N. et. al 2017]. Adherence factors might be responsible for the study patients failing second-line ART rather than HIV strains resistant to ARV drugs, which corroborates to observation among programmatic cohorts in resource limited settings [Eshleman SH. et. al 2004]. HIV drug resistance surveillance outcome among patients failing second-line ART helps in planning evidence driven treatment regimens for patients needing third-line ART. Availability of ARV drugs for those failing second-line ART might be an issue. Failing second line ART that we have encountered here necessitates planning for 3rd line ART program ensuring affordable and accessible supplies of darunavir and raltegravir.

With the introduction of ART, up to 80% decline in rates AIDS associated death has been reported [Bennett DE. et. al. 2009] but it also leads to the steady rise of HIV DR mutant variants that are the major cause of ART failure [Stanford University HIV Drug Resistance Database IVDR; Bakhouch K et.al.2009; Novak RM et.al.2005; Re MC.et.al. 2004]. These variants become predominant over time and pose a major challenge for both ART exposed as well ART naïve patients [Re MC.et.al.2001]. The gradual transmission of resistant mutants from ART-experienced patients to ART naïve individuals was reported from developed countries with even having good access to antiretroviral drugs [Re MC.et.al.2005; Manosuthi W.et.al. 2013; Toni T.et.al. 2007; Bennett DE.et.al.2008]. However, of late, scaling-up of ART lead to the emergence of mutations in resource limited countries too [Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents 2014]. WHO recommends periodic surveillance of transmitted drug resistance (TDR) mutations in ART naïve newly infected PLHIV in distinct geographical areas [Thompson MA.t.al. 2012].

Currently to optimize ART regimen for scaling up, routine assessment of drug resistance-associated mutations prior to ART initiation is recommended where primary resistance has been consistently recorded [Vandamme AM.et.al 2011]. Deficiency in baseline HIVDR data, interruption in treatment and improper administration of drug regimens could be the major factors of occurrence and expansion of drug resistance in resource limited countries.

Conclusion

Nowadays major challenges for anti-retroviral therapy are emergence of HIV drug resistance and virologic failure. Investigations on emergence of HIV DR mutations among patients on ART as well as virologic failure in the absence of HIV DR Mutations might help in better understanding the HIV DR dynamics and planning appropriate clinical management for patients with HIV.

Chapter 5

HIV-1 Diversity among babies (6 weeks to 18 months) born to HIV positive mothers

Preamble

In India approximately 2.39 million people are living with HIV infection which contributes third largest HIV infected population [Suryavanshi N.et.al.2018, Sinha A.et.al.2008]. Vertical transmission from pregnant woman to her new-born during pregnancy in utero, intrapartum during labour and delivery or postpartum through breast feeding plays an important role in India [NACO Guideline 2013] The new born baby receives HIV infection directly from mother but when mother was infected from her partner before conceiving the baby, the Parent to Child (PTCT) is meaningful in this case [NACO Guideline 2013]. In India, 30 million annual pregnancies occur and among those, more than 20,000 pregnant women are infected with HIV, estimated by National AIDS Control Organization [NACO Technical Report 2017]. Transmission of HIV infection occurs 70 – 75% at the time of delivery or labour where as in the late stage of pregnancy 25 – 30% transmission occurs [Irene YV.et.al.2010]. In eastern part of India, prevalence of HIV infection due to mother to child transmission (MTCT) recorded highest 29.41% [Mandal S.et.al.2010]. MTCT plays crucial role for persisting HIV infection as children contribute 7% of new HIV infection [Nath A.et.al.2017]. PMTCT intervention is initiated in India in 2002, where all the pregnant women diagnose for HIV infection with free of cost at the integrated counseling and Testing Centre (ICTC) and after detection of HIV positivity, the mother is enrolled for getting free Anti-retroviral Drug (ARV) lifelong and to the children immediate after birth to prevent vertical transmission [Dhadwal D.et.al.2009]. In India, WHO recommended ARV drugs as a prophylaxis strategy has been adopted by NACO that results the improvement of health of pregnant women, prevent vertical transmission and thus enable "Early Infant Diagnosis [World Health Organization. PMTCT strategic vision 2010–2015, WHO. Global guidance on criteria and processess for validation, 2017].

This study aimed to generate data on the subtype diversity and in addition drug resistance to HIV among the babies (6 weeks to 18 months) born to HIV positive mother in India.

Material and Methods

Study Population and setting

The present study was conducted at ICMR-national Institute of Cholera and Enteric Diseases, Kolkata. Babies, 6 weeks to 18 months of age born to HIV positive mothers were recruited in the present study over the period of two.years. The diagnosis of HIV-1 infection in infants and children less than 18 months of age (starting at 6 weeks of age) is the HIV-1 DNA PCR test. Here HIV-1 is detected using real time PCR, where mainly proviral DNA of virus is detected in babies.

A total of 2633 infants were tested for HIV-1 DNA PCR and among those were detected positive were included for further processing in the study.

Data Collection

Basic socio-demographic data (age, sex, address, mother's ART no) with current feeding practice of the child were collected. As the vertical transmission of HIV contributes a major role in HIV prevalence, the breastfeeding status plays an important part within it. The data has been collected through an appropriate set of previously prepared questionnaires by an experienced Counselor.

Specimen collection and processing

In the present study the Dried Blood Spots were used for following advantages:

- Requires only a small amount of blood (30-100µl per spot)
- Easy to store

- Easy to transport
- Low biohazard
- Usually obtained from pricking skin
- A widely used method of specimen collection for HIV-1 PCR testing

A spot of blood from a requisite site according to their age was placed on filter paper and allowed to air dry. Infants ages between 6 weeks to 18 months, heel prick; big toe was recommended for 4 to 10 months of age and 3rd and 4th finger were used for the infants ages between 10 to 18 months. A good DBS card should be properly dried and dark brown in colour with minimum 3 good blood spots and labeled with proper identification code and date and time of collection. A circular punch (about 12mm) is removed, eluted with the solvent and analyzed for metabolic markers.

Detection of HIV positivity status of the infants

HIV-1 DNA PCR was used to detect the positivity status of infants. The test was performed in automated close system; Abbott m2000sp and Abbott m2000rt.

The Abbott Real time HIV-1 Qualitative Assay detects HIV-1 nucleic acids by using Polymerase Chain Reaction (PCR) technology with homogenous real-time fluorescent detection. Partially double-stranded fluorescent probe design allows detection of diverse HIV-1 groups and subtypes. The assay uses either human plasma or DBS specimen type and reports a qualitative result.

Sample preparation from DBS specimen

The purpose of sample preparation is to extract, concentrate, and purify the target nucleic acids for amplification. The Abbott sample Preparation System DNA uses magnetic particle

technology to capture target nucleic acids and washes the particles to remove unbound sample components. The bound nucleic acids are eluted and are then ready for amplification. The IC is taken through the entire sample preparation procedure along with the controls and specimens. Sample can be prepared manually. The sample preparation process consists of the following:

- Releasing the nucleic acids from its native biological source (e.g. lysis of cells)
- Binding of nucleic acid to solid phase (i.e. the magnetic particles)
- Separation of solid phase from residual lysis solution
- Washing to remove unwanted particles
- Elution or separation of nucleic acid from solid phase.

1. A maximum of 96 samples can be processed per run. A negative control and a positive control are included in each run, therefore allowing a maximum of 94 DBS specimens to be processed per run. Process the DBS specimens by following these steps:

- Two entire DBS (12 mm in diameter) for each specimen from a Whatman903 filter paper card (or equivalent) is cut with help of a DBS puncher and placed in polypropylene tubes (Falcon tube).
- 1.7 ml Abbott $mLysis_{DNA}$ Buffer (from the bulk $mLysis_{DNA}$ buffer) is added to each tube and ensured that the DBS are fully submerged in the $mlysis_{DNA}$ buffer.
- Incubated at room temperature for 20 min with intermittent gentle mixing (generally on platform shaker).
- After incubation all the liquid is carefully pipette out to a 5ml reaction vessel avoiding transfer of bubbles. Filter paper (DBS) is not transferred.

Reagent preparation

1. Assay control and IC are thawed at 15-30°C or at 2-8°C.

- Each assay control is vortexed 3 times for 2 to 3 seconds before use. then it is ensured that the contents of each vial are at the bottom after vertexing by tapping the vials on the bench to bring liquid to the bottom of the vial. It should also be ensured that the bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

2. After opening the mSample Preparation System$_{DNA}$ reagent pack, Ethanol (95 to 100% Ethanol) to the mLysisDNA, mWash 1DNA, and mWash 2DNA bottles as indicated below.

- Add 35 mL ethanol to each bottle of mLysis$_{DNA}$ being used.
- Add 23 mL ethanol to each bottle of mWash 1$_{DNA}$ being used.
- Add 70 mL ethanol to each bottle of mWash 2$_{DNA}$ being used.

3. Each IC vial is Vortexed 3 times for 2 to 3 seconds before use, Using a calibrated precision pipette to add 750 µL of IC to each bottle of mLysis$_{DNA}$ Buffer.

4. All the reagent bottles except mMicroparticles$_{DNA}$ 5 to 10 times to ensure a homogenous solution and the contents are poured into the appropriate reagent vessels. Ensure bubbles or foam are not generated in the reagent vessels; if present, remove with a sterile pipette tip, using a new tip for each reagent vessel.

5. The negative control, positive control, and the patient specimens are placed into the Abbott m2000sp sample rack.

6. 5 ml Reaction Vessels and 96 deep-well plate are loaded into the Abbott m2000sp 1 mL subsystem carrier.

7. From the protocol screen, appropriate application file is selected and the sample extraction protocol is initiated.

Sample Extraction

The procedure of sample extraction done in the m2000sp constitutes the following processes:-

- 5ml reaction vessels are placed in the non-magnetic rack at room temperature.
- 40µl of mMicroparticles$_{DNA}$ is added to each reaction vessel.
- 2.4ml of mLysis$_{DNA}$ is added to each reaction vessel.
- 1.0ml of controls and specimens are added to the reaction vessels. The sample-mLysis$_{DNA}$ mixture is mixed by aspiration and dispensed until a uniform suspension is obtained.
- Reaction vessels are placed in the 50°C heating block and incubated for 20 minutes.
- Reaction vessels are removed from the 50°C heating block and the sample-mLysis$_{DNA}$ mixture is mixed by aspiration and dispensed until a uniform suspension is obtained.
- Reaction vessels are placed in the 50°C heating block for 10 minutes.
- After the incubation is complete, reaction vessels are placed in the magnetic capture stand for 2 minutes to allow the particles to be captured on the side of the reaction vessels.
- With the reaction vessels in the magnetic capture stand, the lysate is removed from each reaction vessel and the fluid is discarded into the liquid waste container. The fluid is removed as completely as possible without disturbing or aspirating the captured magnetic particles.

- Reaction vessels are removed from the magnetic and transferred to the non-magnetic rack.

mWash 1 Buffer$_{DNA}$ steps

- 750µl of mwash 1_{DNA} was added to the samples and the magnetic particles re-suspended in the wash fluid by gentle aspiration.
- Reaction vessels are placed in the 50°C heating block and incubated for 5 minutes.
- Reaction vessels are removed from the 50°C heating block and the sample-mWash 1_{DNA} mixture is mixed by aspiration and dispensed until a uniform suspension is obtained.
- Reaction vessels are placed in a magnetic capture stand for one minute to allow the particles to be captured on the side of the tubes.
- With the reaction vessels in the magnetic capture stand, the mWash1_{DNA} is removed from each reaction vessel as completely as possible without disturbing or aspirating the captured magnetic particles.
- The reaction vessels are removed from the magnetic rack and the transfer to the non-magnetic rack.

mWash 2$_{DNA}$ First Wash

- 800µl of mWash 2_{DNA} is added to the samples and the magnetic particles are re-suspended in the wash fluid by gentle aspiration.
- Reaction vessels are placed in the magnetic capture stand for one minute to allow the particles to be captured on the side of the tubes.

- With the reaction vessels in the magnetic capture stand, the mWash $1_{DNA\ is}$ removed from each reaction vessel and the fluid discarded into the liquid waste container. The fluid is removed as completely as possible without disturbing as aspirating the captured magnetic particles.
- Reaction vessels are removed from the magnetic rack and transferred to the non-magnetic rack.

mWash 2_{DNA} Second Wash

- 800µl of mWash $2_{DNA\ \mathbf{is}}$ added to the samples and the magnetic particles are re-suspended in the wash fluid by gentle aspiration.
- Reaction vessels are placed in the magnetic capture stand for one minute to allow the particles to be captured on the side of the tubes.
- With the reaction vessels in the magnetic capture stand, the mWash 2_{DNA} is removed from each reaction vessel and the fluid discarded into the liquid waste container. The fluid is removed as completely as possible without disturbing or aspirating the captured magnetic particles.
- Reaction vessels are removed from the magnetic rack and transferred to the 75°C heating block and incubated.

mElution Buffer$_{DNA}$

- Removing the tubes from the 75°C heating block, 88µl of mElution Buffer$_{DNA}$ is added to the samples and the magnetic particles re-suspended in the fluid by aspiration and dispensed.
- Reaction vessels are placed in the 75°C heating block and incubated for 15 minutes.

- Reaction vessels are removed from the 75°C heating block and the sample-mElution buffer $_{DNA}$ mixture is mixed by aspiration and dispensed until a uniform suspension is obtained.
- Reaction vessels are placed back again in the 75°C heating block and incubated for 5 minutes.
- Reaction vessels are removed from the 75°C heating block and placed in magnetic capture stand for one minute to allow the particles to be captured on the side of the tubes.
- With the reaction vessels in the magnetic capture stand, the eluted sample is removed from the tubes without disturbing or aspirating the captured microparticles. The eluted samples are placed in the 96-well polypropylene plate.

Amplification reagent addition

- The amplification reagents and the master mix tube on the Abbott m2000sp worktable after sample preparation is completed. The 96- well optical reaction plate is also loaded into the cooler plate at 8°C on the m2000sp worktable. Each Amplification reagent pack supports up to 24 reactions.
- From the protocol screen, the Abbott m2000sp Master mix addition protocol is initiated. In the meantime, the Abbott m2000rt instrument is switched on and initialized.

The procedure of amplification reagent mixture goes through the following steps:

- 271µl of the HIV-1 Activation reagent and 949µl of the HIV-1 Oligonucleotide Reagent are added to the thermo stable rTth DNA polymerase enzyme bottle. The mixture is mixed properly by aspiration and dispensed gently.

- Contents of the master mix from the enzyme bottle are pipette into the master mix tube (RNase/DNase-free tube) and mixed gently by aspiration and dispensed.
- 50µl aliquots of the amplification master mix are dispensed into each well of the Abbott 96-well optical reaction plate.
- 50µl of each eluted sample is transferred to the Abbott 96- well optical reaction plate on the cooler plate (being set at 8°C). during the transfer of each sample, the reaction mixture is mixed by pipetting up and down three to five times.
- Abbott 96-well optical reaction plate is sealed and placed in the Abbott m2000rt instrument.
- Test order is selected and the Abbott Real-Time HIV-1 quality assay protocol is initiated.
- At the completion of the run, assay results are reported on the Abbott m2000rt.

HIV-1 Genotyping

All the samples detected as positive by qualitative PCR, were undergone for HIV-1 genotyping.

DBS preparation

By using a puncher, the dried blood spot was punched without touching the spotted blood.

The blood spot was added to a 2ml tube containing lysis buffer.

The tubes were incubated for 30 minutes at room temperature.

Centrifugation was done for 15 seconds at 1500 x g.

The lysate was transferred for nucleic acid extraction.

RNA Extraction and Purification

- Extraction of RNA and purification has been done by using QIAamp Viral RNA mini kit followed by manufacturer's instruction.
- QIAamp Viral RNA Mini Kits represents a well-established technology for general use viral RNA preparation. The kit combines the selective binding properties of a silica based membrane with the speed of microspin.
- Lysis: The sample is first lysed under the highly denaturing conditions provided by buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to buffer AVL, improves the binding of viral RNA to the QIAamp membrane.
- Then the sample was loaded onto the QIAamp Mini Spin column.
- The RNA binds to the membrane, and contaminants efficiently washed away in 2 steps using 2 different wash buffer.
- High quality RNA is eluted in a special RNase free buffer, ready for direct use or safe storage.
- The purified RNA is free of protein, nucleases and other contaminants and inhibitors.

Amplification

RT-PCR

HIV-1 pol gene was amplified by one tube reverse transcriptase Polymerase Chain Reaction (RT-PCR). The total procedure has been carried out by using Qiagen RT-PR Mini kit (Qiagen, GmBH, Hilden, Germany) manufacturer's instructions.

The procedure was carried out by round -1 RT-PCR followed by nested PCR.

RT-PCR master mix is prepared as per Table 2.1, Chapter 2 followed by proper vortexing and centrifugation as per standard protocol described in the manufacturer's instructions.

In 200 µl MicroAmp reaction tube, 10 µl of RNA extract was added and similarly addition of positive and negative control was done.

The following program was carried out in the thermal cycler as per thermal cycling profile described in Table 2.2, chapter 2.

Inside the laminar airflow, 15 µl RT-PCR brewmix was added to each tube. The RT-PCR master mix contains RNase free water, 5x Qiagen One Step RT-PR buffer (contains 12.5mM $MgCl_2$), 2021 F primer, 4521 R primer, dNTP mix, RNAse inhibitor, QIAGEN One step RT PCR enzyme mix, RNA Template.

The thermal cycler was turned on and QIA-RT-PCR program was run (Refer Table 2.3, Chapter 2).

The thermal cycling profile for RT-PCR and Nested PCR were tabulated in Table 2.3 and Table 2.5, chapter 2.

Nested – PCR Amplification:

- In a 1.5 ml micro centrifuge tube, the following reagents were added in order: Taq DNA polymerase (Genei, Bangalore, India), 3 U/ µl (1 µl) in a 10x PCR buffer B (5 µl), 25 mM $MgCl_2$ (4 µl), 2mM dNTPs Mix (5 µl), 10 pmol of each primer and 6 µl of RT-PCR product, The final volume of 50 µl reaction was made up by using DNase / RNase free water. The preparation of Nested- PCR master mix was described in Table 2.4, chapter 2.
- After centrifugation, 44 µl of PCR master mix was pipetted out and 6 µl of RT-PCR product was added to Micro Amp sample tube.

- Nested-PCR reaction was performed in the thermal cycler using the prescribed program.
- The primer used for amplification of protease and RT region of HIV-1 were described in Table 2.6, chapter 2

Gel Electrophoresis and purification

5 - 10 µl nested-PCR products were checked by electrophoresis at 100V on 1% agarose gel in 1X TAE buffer along with standard molecular weightmarker. Ethidium bromide solution was used as staining solution for 30 mins and visualized under UV-illuminator.. The PCR product was purified by using commercially available PCR purification kit.

Sequencing PCR Product:

- Before starting the procedure, the purified PCR product was diluted to 30 ng/µl (15 – 25 µl total volume) using DEPC treated water.
- The sequencing PCR was done by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystem, CA, USA). The Big Dye chemistry was carried out with HIV-SEQ mix and diluted purified PCR product with final reaction volume 10 µl. In-house HIV-SEQ mix was prepared followed by Table 2.7, chapter 2
- Diluted, purified PCR products and the sequencing primers were thawed.
- HIV Seq mixes was pulse vortexed.
- To the appropriate well of the plate, 8 µl HIV Seq mix were added followed by necessary precautions as per manufacturer's instruction.
- PCR product was vortexed. 2µl diluted, purified PCR product was added to the well into the plate.

- Centrifugation and spinning was done as per manufacturer's instruction
- Cover the plate with a rubber 96 well plate septa.
- Incubate sequencing Reactions in the thermal cycler followed by program tabulated in Table 2.8, chapter 2.
- Here the primer used for HIV-1 sequencing PCR were same as described in Table 2.9 of chapter 2.

Post Sequencing PCR Precipitation:

- Using 200µl multichannel pipettor, 80µl of 80% isopropanol to each 10 µl sequencing reaction was added.
- The plate was covered immediately with septa
- Mixing was done by inverting the plate three times.
- The plate was incubated for 15minutes at room temperature in dark.
- Centrifugation was done for 45 minutes at 35R at 2000 x g
- After removing the cover, the plate was inverted on a folded tissue paper and centrifugation was done at 700 g x for 1 minute.
- The plate was dried for 5 minutes
- The samples were resuspended in 20µl of HiDi Formamide cover with septa.
- Finally, the plate was loaded into the sequencer for sequencing.

Analysis

ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA): The PCR products were sequenced on a 16 capillary automated florescence based Genetic

Analyzer. ABI data collection software was used to program the sequence analysis file. The Sequence Analysis Software v5.3 was used to extract base calling sequenced data.

Sequence Analysis: The ABI files for each sample were analyzed by using ABI Sequence Analysis Software v5.3.

The forward and reverse sequences for each sample were assembled and edited with wild type reference sequence HXB-2 where multiple sequence alignment Secscape software was used and the FASTA sequences were exported.

HIV Drug Resistance Algorithm: The obtained DNA sequences were submitted to HIV drug resistance database of Stanford University (http://hivdb.stanford.edu/hiv). Subtyping of obtained sequences was performed through comparing them with reference sequence through Basic Local Alignment Search Tool (BLAST).

Quality Control

A previously identified high positive and negative samples run as control in every bath. The successful result of positive control ensures the effective RT PCR & genotyping. For the quality assurance of present sequences, a known positive sample was sequenced as control prior to the clinical samples of this study.

Clade typing

HIV-1 subtyping has been done by using REGA HIV-1 subtyping tool from the HIV drug resistance database of Stanford University. All the sequences of the present study were compared with the HIV database of Los Alamos.

Results

The study was conducted at division of virology, ICMR-National Institute of Cholera and Enteric Diseases, a NABL accredited laboratory that maintains international standard, ISO 15189:2012. In the present study, over the period of two years, total 2633 subjects were recruited and tested. The inclusion criteria were only the new born babies aged 6 weeks to 18 months and born to HIV infected mother.

Irrespective of any selection bias, the subjects was included consecutively over the period of two years. Among total recruited samples, 193 samples were detected as HIV-1 DNA PCR positive which has been sequenced further for detection of subtype and drug resistance mutation testing.

Among these total 193 samples, 183 samples were successfully sequenced and the data was analyzed. Rest of 10 samples were not sequenced may be due to present of any inhibitor in the DBS card or contains very low virus copy numbers.

Among these 183 sequenced samples, more than 90% samples are detected as HIV-1 subtype C. Rest of the samples are identified as HIV-1 subtype D and subtype J (Fig: 1). Among these HIV-1 subtype C, 10 samples are spotted as recombinant of subtype C. Recombinant of C and J found in three samples; recombinant of C, A1 spotted in two samples; recombinant of C, G, A1 were marked in two samples and recombinant of 07_BC, D was detected in one sample.

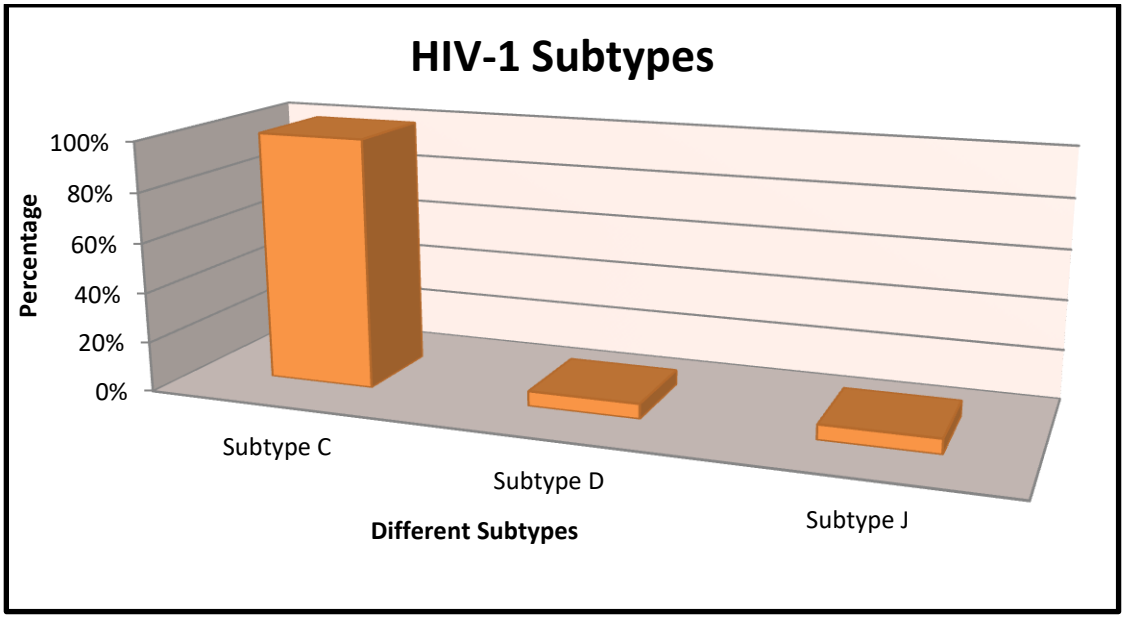

Figure 5.1: HIV-1 subtype diversity among study subjects

Table 5.1: Details of individual samples for subtype C diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI002	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI003	HIV-1 Subtype C	HIV-1 Subtype C	2082	3393	1300
DBS MI004	HIV-1 Subtype C	HIV-1 Subtype C	2252	3565	1313
DBS MI005	HIV-1 Subtype C	HIV-1 Subtype C	2082	3393	1300
DBS MI007	HIV-1 Subtype C	HIV-1 Subtype C	2252	3551	1310
DBS MI008	HIV-1 Subtype C	HIV-1 Subtype C	2252	3580	1328
DBS MI009	HIV-1 Subtype C	HIV-1 Subtype C	2296	3562	1269
DBS MI010	HIV-1 Subtype C	HIV-1 Subtype C	2252	3566	1322
DBS MI011	HIV-1 Subtype C	HIV-1 Subtype C	2252	3568	1322
DBS MI012	HIV-1 Subtype C	HIV-1 Subtype C	2252	3584	1332
DBS MI013	HIV-1 Subtype C	HIV-1 Subtype C	2216	3476	1262
DBS MI014	HIV-1 Subtype C	HIV-1 Subtype C	2252	3542	1290
DBS MI015	HIV-1 Subtype C	HIV-1 Subtype C	2252	3512	1261
DBS MI016	HIV-1 Subtype C	HIV-1 Subtype C	2252	3602	1350
DBS MI017	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1304
DBS MI018	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1314
DBS MI019	HIV-1 Subtype C	HIV-1 Subtype C	2203	3558	1351
DBS MI020	HIV-1 Subtype C	HIV-1 Subtype C	2236	3575	1339
DBS MI021	HIV-1 Subtype C	HIV-1 Subtype C	2236	3557	1321
DBS MI022	HIV-1 Subtype C	HIV-1 Subtype C	2240	3575	1334
DBS MI023	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI024	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI025	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI026	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI028	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI029	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI030	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI031	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI032	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI033	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI034	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI035	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI036	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI037	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI038	HIV-1 Subtype C	HIV-1 Subtype C	7142	8309	1164
DBS MI041	Recombinant of 07_BC, D	HIV-1 Subtype C	6267	7655	1382
DBS MI043	Recombinant of C, A1	HIV-1 Subtype C	2819	4129	1310
DBS MI044	HIV-1 Subtype C	HIV-1 Subtype C	2168	3552	1384
DBS MI045	HIV-1 Subtype C	HIV-1 Subtype C	1877	3191	1312
DBS MI046	HIV-1 Subtype C	HIV-1 Subtype C	2193	3554	1361
DBS MI047	HIV-1 Subtype C	HIV-1 Subtype C	2809	4147	1338

Table 5.1: Details of individual samples for subtype C diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI048	HIV-1 Subtype C	HIV-1 Subtype C	1866	3176	1310
DBS MI049	HIV-1 Subtype C	HIV-1 Subtype C	2348	3698	1348
DBS MI051	HIV-1 Subtype C	HIV-1 Subtype C	1982	3313	1331
DBS MI052	Recombinant of C, J	HIV-1 Subtype C	1956	3286	1330
DBS MI055	HIV-1 Subtype C	HIV-1 Subtype C	2240	3575	1334
DBS MI056	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI057	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI058	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI059	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI061	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI062	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI063	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI064	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI065	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI066	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI067	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI068	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI069	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI070	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI071	HIV-1 Subtype C recombinant	HIV-1 Subtype C	7142	8309	1164
DBS MI074	Recombinant of C, A1	HIV-1 Subtype C	2819	4129	1310
DBS MI075	HIV-1 Subtype C	HIV-1 Subtype C	2168	3552	1384
DBS MI076	HIV-1 Subtype C	HIV-1 Subtype C	1877	3191	1312
DBS MI077	HIV-1 Subtype C	HIV-1 Subtype C	2193	3554	1361
DBS MI078	HIV-1 Subtype C	HIV-1 Subtype C	2809	4147	1338
DBS MI079	HIV-1 Subtype C	HIV-1 Subtype C	1866	3176	1310
DBS MI080	HIV-1 Subtype C	HIV-1 Subtype C	2348	3698	1348
DBS MI081	HIV-1 Subtype C	HIV-1 Subtype C	1982	3313	1331
DBS MI082	HIV-1 Subtype C	HIV-1 Subtype C	1956	3286	1330
DBS MI085	HIV-1 Subtype C	HIV-1 Subtype C	2240	3575	1334
DBS MI086	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI090	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI091	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI092	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI093	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI094	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI095	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307

Table 5.1: Details of individual samples for subtype C diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI097	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI098	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI099	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI100	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI101	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI102	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI103	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI104	HIV-1 Subtype C	HIV-1 Subtype C	2819	4129	1310
DBS MI105	HIV-1 Subtype C	HIV-1 Subtype C	2168	3552	1384
DBS MI106	HIV-1 Subtype C	HIV-1 Subtype C	1877	3191	1312
DBS MI107	HIV-1 Subtype C	HIV-1 Subtype C	2193	3554	1361
DBS MI108	HIV-1 Subtype C	HIV-1 Subtype C	2809	4147	1338
DBS MI109	HIV-1 Subtype C	HIV-1 Subtype C	1866	3176	1310
DBS MI110	HIV-1 Subtype C	HIV-1 Subtype C	2348	3698	1348
DBS MI111	Recombinant of C, G, A1	HIV-1 Subtype C	1982	3313	1331
DBS MI112	Recombinant of C, J	HIV-1 Subtype C	1956	3286	1330
DBS MI115	HIV-1 Subtype J recombinant	HIV-1 Subtype C	2240	3575	1334
DBS MI116	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI117	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI118	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI119	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI120	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI121	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI124	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI125	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI126	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI127	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI128	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI129	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI130	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI131	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI132	HIV-1 Subtype C	HIV-1 Subtype C	2819	4129	1310
DBS MI133	HIV-1 Subtype C	HIV-1 Subtype C	2168	3552	1384
DBS MI134	HIV-1 Subtype C	HIV-1 Subtype C	1877	3191	1312
DBS MI135	HIV-1 Subtype C	HIV-1 Subtype C	2193	3554	1361
DBS MI136	HIV-1 Subtype C	HIV-1 Subtype C	2809	4147	1338
DBS MI137	HIV-1 Subtype C	HIV-1 Subtype C	1866	3176	1310

Table 5.1: Details of individual samples for subtype C diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI138	HIV-1 Subtype C	HIV-1 Subtype C	2348	3698	1348
DBS MI140	Recombinant of C, G, A1	HIV-1 Subtype C	1982	3313	1331
DBS MI141	Recombinant of C, J	HIV-1 Subtype C	1956	3286	1330
DBS MI144	HIV-1 Subtype C	HIV-1 Subtype C	2240	3575	1334
DBS MI145	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI146	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI147	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI148	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI149	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI150	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI151	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI152	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI153	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI154	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI155	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI156	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI157	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI158	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI159	HIV-1 Subtype C	HIV-1 Subtype C	2819	4129	1310
DBS MI160	HIV-1 Subtype C	HIV-1 Subtype C	2168	3552	1384
DBS MI161	HIV-1 Subtype C	HIV-1 Subtype C	1877	3191	1312
DBS MI162	HIV-1 Subtype C	HIV-1 Subtype C	2193	3554	1361
DBS MI163	HIV-1 Subtype C	HIV-1 Subtype C	2809	4147	1338
DBS MI164	HIV-1 Subtype C	HIV-1 Subtype C	1866	3176	1310
DBS MI166	HIV-1 Subtype C	HIV-1 Subtype C	2348	3698	1348
DBS MI167	HIV-1 Subtype C	HIV-1 Subtype C	1982	3313	1331
DBS MI168	HIV-1 Subtype C	HIV-1 Subtype C	1956	3286	1330
DBS MI169	HIV-1 Subtype C	HIV-1 Subtype C	6337	7666	1329
DBS MI170	HIV-1 Subtype C	HIV-1 Subtype C	6958	8255	1291
DBS MI172	HIV-1 Subtype C	HIV-1 Subtype C	2240	3575	1334
DBS MI173	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI174	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI176	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI177	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI178	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI179	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI180	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307

Table 5.1: Details of individual samples for subtype C diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI181	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI182	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI183	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI185	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202
DBS MI186	HIV-1 Subtype C	HIV-1 Subtype C	2252	3552	1300
DBS MI187	HIV-1 Subtype C	HIV-1 Subtype C	2136	3369	1233
DBS MI188	HIV-1 Subtype C	HIV-1 Subtype C	2253	3552	1299
DBS MI189	HIV-1 Subtype C	HIV-1 Subtype C	2252	3556	1304
DBS MI190	HIV-1 Subtype C	HIV-1 Subtype C	2248	3444	1196
DBS MI191	HIV-1 Subtype C	HIV-1 Subtype C	2248	3561	1309
DBS MI192	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI194	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI195	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI196	HIV-1 Subtype C	HIV-1 Subtype C	2252	3559	1307
DBS MI197	HIV-1 Subtype C	HIV-1 Subtype C	2252	3555	1303
DBS MI198	HIV-1 Subtype C	HIV-1 Subtype C	2252	3558	1306
DBS MI199	HIV-1 Subtype C	HIV-1 Subtype C	2252	3554	1302
DBS MI200	HIV-1 Subtype C	HIV-1 Subtype C	2252	3452	1202

Table 5.2: Details of individual samples for subtype D diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI039	HIV-1 Subtype D	HIV-1 Subtype D	6363	7593	1224
DBS MI053	Recombinant of G, C, D	HIV-1 Subtype D	6337	7666	1329
DBS MI073	HIV-1 Subtype D	HIV-1 Subtype D	6363	7593	1224
DBS MI083	Recombinant of C, D	HIV-1 Subtype D	6337	7666	1329
DBS MI113	Recombinant of G, C, D	HIV-1 Subtype D	6337	7666	1329
DBS MI142	Recombinant of G, C, D	HIV-1 Subtype D	6337	7666	1329

Table 5.3: Details of individual samples for subtype J diversity among infants

Sample Code	HIV-1 Subtype (assignment)	HIV-1 Subtype (Pure)	Begin	End	Length (kb)
DBS MI040	HIV-1 Subtype J recombinant	HIV-1 Subtype J	7001	8408	1398
DBS MI042	Recombinant of A1, C	HIV-1 Subtype J	6530	7897	1361
DBS MI054	Recombinant of J, D	HIV-1 Subtype J	6958	8255	1291
DBS MI084	Recombinant of J, D	HIV-1 Subtype J	6958	8255	1291
DBS MI114	Recombinant of J, D	HIV-1 Subtype J	6958	8255	1291
DBS MI143	Recombinant of J, D	HIV-1 Subtype J	6958	8255	1291

Here among these 3.30% HIV-1 subtype D detected samples, recombinant of C, D was found in one sample whereas recombinant of G, C, D were identified in three samples.

HIV-1 subtype J has been detected in 3.30% samples and among which recombinant of A1, C was marked in one sample. Recombinant of J, D was identified in four samples. The length of the sequencing genome is in between 1164 – 1384bp (Table 5.1, Table 5.2 and Table 5.3).

The present study reveals no drug resistance mutations among all the successfully sequenced samples.

Phylogenetic Analysis

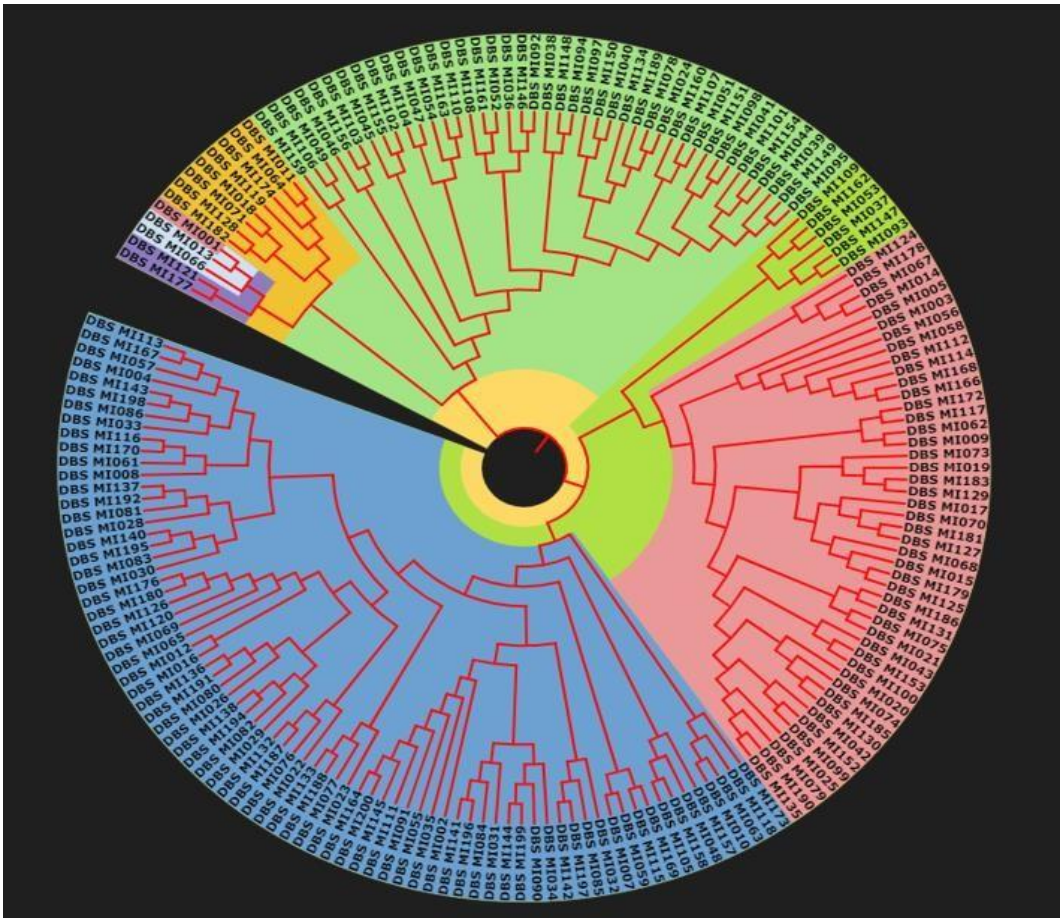

Figure 5.2: Molecular Phylogenetic analysis by Maximum Likelihood method

Divergence Times for all branching points in the topology were calculated using Maximum Likelihood Method based on the Tamura-Nei Model. Bars around each node represent 95% confidence intervals which were computed using the method in published data [Tamuraet.et.al.1993, Tamuraet.et.al.2012]. The tree is drawn to scale, with branch lengths

measured in the relative number of substitutions per site. The analysis involved 183 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. Ll positions containing gaps and missing data were removed. The Evolutionary analysis was conducted in MEGA6 [Tamuraet.et.al.2013]

Discussion:

The primary route of HIV transmission among adults is mainly by sexual intercourse whereas the parent to child transmission plays a large incident from pregnant woman to her new born during pregnancy [Damania KR.et.al 2006]. As per estimation by National AIDS Control Organization (NACO), that the annual pregnancy in India is about 30 million whereas the pregnant women are infected by HIV is 22,000 [NACO annual technical report]. From 2016, free diagnosis of HIV has been made available through Integrated Counseling and Testing Centers (ICTCs). This service indirectly prevents the prenatal transmission from pregnant woman with HIV to her new born. In India, NACO initiated to implement "Option B+" PMTCT facility. This facility includes providing lifelong ART to HIV infected woman with pregnancy to avoid vertical transmission and administration nevirapine for 6 to 12 weeks HIV infected breastfeeded children with engagement and retention of mother and baby in post-partum care of HIV which enable early infant diagnosis [WHO Global guidance 2017]. In India, HIV transmission from mother to her newborn was ranging from as low as 3.49% to as high as 29.4% and both the studies were carried out in the West Bengal [Chaudhuri S.et.al. 2010 and Mandal S.et.al.2010]. The present study findings corroborate with these published articles from eastern region of India.

HIV is a genetically diverse lentivirus and poses variety of subtypes along with inter-subtype recombinants. Subtype C collectively predominates in Southern Africa [Sivay MV.et.al.2018]. An increase in pure subtype C compared with subtype B has also been

reported among HIV/viral hepatitis co-infected patients in Southern Brazil [Avanzi VM.et.al.2017]. Subtype C predominates in India with reports of increased UFRs in the north eastern part of the country [Sharma AL.et.al.2017]. The present study revealed and supports these studies by estimating more than 90% subtype C in the eastern part of India.

An increase in subtype C and decrease in subtype D have been reported in Kenya with inter-subtype recombination [Gounder K.et.al.2017]. The present study also corroborates the previously published articles by presenting about 3% subtype D diversity among study population.

In mother to child transmission, the maternal antibodies passively transferred to the child but not all the children get infection from mother. This is may be due to intervention of ART and proper HIV-1 clinical management [Doepker LE.et.al. 2020]. Similarly, in the present study, transmission of HIV from infected mother to her newborn is very less. This study also revealed no HIV-1 drug resistance mutation among the infected children those got the HIV infection from their mother. The prior studies describe the ability of ART for prevention of infection in new cells. The in vivo reproductive rate of the virus seems to be reduced less than 1 and viral evolution has been halted effectively by ART where preventing of associated development of drug resistance occur simultaneously [Kearney MF, et.al. 2014]. A study among HIV-1 infected children with low viral diversity also divulges no evidence of HIV replication in children on antiretroviral therapy [Van Zyl GU.et.al.2017]

Conclusion

HIV is one of the most genetically diverse viruses due to its high-mutation, recombination, and rapid replication rate. This rapid evolutionary process has resulted in several HIV subtypes that are heterogeneously distributed around the world. Subtype A generally found in parts of East Africa, Russia and former Soviet Union countries, subtype B found in

European countries along with USA and Australia, subtype C found in Southern Africa and South-east Asia including India; CRF02_AG in Western Africa, CRF03_AB in middle East. Recent works on near full-length genome sequencing of HIV-1 shows increasing events of different recombinant variants and subtype C viruses. The active change in HIV-1 subtype distribution patterns initiate global challenges for diagnosis, treatment and ARV drug design. Increase in recombinant viruses suggests that coinfection and superinfection by divergent HIV strain has become more common. At present near full length genome sequencing is essential for improving HIV-1 subtypes estimates that would help for better clinical management.

Chapter 6

Summary and scope of Future Work

Summary

Studies on genetic diversity of HIV are hardly reported in India though it harbors a largest pool of HIV infected individuals globally. Therefore, understanding HIV diversity might help in planning effective intervention program for prevention and control of HIV infection, a colossal public health challenge.

In chapter 2, the study shows the pattern of HIV drug resistance in people living with HIV (PLHIV) under pretreatment. As per data collected from the study participants of present study, no previous exposure to ART has been ensured by the participants as they have first time detected as HIV positive in this center. So, pretreatment drug resistance has been found in the patients before they begin their treatment.

In any treatment, it is important to take all medicine regularly. But sometimes it is difficult as people cannot stick into the treatment when they need it the most. So when they don't take the medicine regularly, the virus may become resistance. In this scenario, most effective regimen or effective medicine can stop to work because the virus stops to respond in that treatment. As a consequence, the people at that time switch to different drugs and those drugs may harder to obtain, may be very expensive and may have the side effect.

As per WHO, at the end of 2020, 27.5 million people were receiving anti-retroviral therapy worldwide. The effectiveness of anti-retroviral drugs in reducing HIV incidence and HIV associated morbidity and mortality can be compromised by HIV drug resistance.

In the present study we have found variation of major and accessary PI mutation, NNRTI mutation, NRTI mutation in the ART naïve PLHIV that directly support WHO findings of HIV drug resistance to NNRTI drug class can have up to 10% among adults starting HIV treatment.

In chapter 3, the study indicates the high prevalence in HIV drug resistance mutations among ART patients from eastern India with 1st line ART failure. This kind of study is hardly reported in India. In management of HIV infection, introduction of ART plays major role worldwide. In India, after initiation of National AIDS Control Program by National AIDS Control Organization, access to ART for general population was increased remarkably. At the same time, poor adherence was observed among the people receiving ART. Treatment failure is diagnosed by virological failure, immunological failure and clinical failure. The virological failure is measured as plasma viral load is more than 1000 copies/ml; whereas immunological failure is referred as CD4 cell count is < 200/ µl and clinical failure is recognized as appearance of WHO stage 3 or stage 4 manifestation after six months ART initiation. Viral load estimation is primary diagnostic tool for early detection of treatment failure. But in India, detection of ART failure is based on clinical or immunological failure because of resource limited settings. So, chances of development of HIV drug resistance is increased because of late switching to first line ART to second line regimen. With the successful ART regimen program, simultaneously HIV drug resistance testing as per WHO guidelines for early detection is highly recommended. The finding of this study will help in effective future planning and establish strategies in therapeutic regimen of treatment failure.

In chapter 4,

This chapter aimed to determine virological failure on anti-retroviral therapy among patients without drug resistance mutations.

HIV produces billions of virions daily through their replication cycles. Mutations occur at high rate at the reverse transcription stage of HIV replication process. The patients with good adherence to ART maintain suppressed HIV RNA level that cause insufficient HIV replication. Inadequate adherence to ART develops mutations and dominant resistance strains

of HIV that can results a suboptimal response to antiretroviral therapy and virologic failure which is referred to as acquired resistance.

The present study find out drug resistance to five patients among nine patient samples tested. Four patients have shown no HIV drug resistance mutation. All the patients in this study received second line ART. The criteria for shifting first line ART to second line ART are treatment failure in clinical or immunological or virological parameter of patients in first line ART for last six months. The patients with drug resistance mutation in second line ART, shows necessity for shifting from second line ART to third line ART.

Non-adherence to ART is an intricate and multi-dimensional challenge. As per published data, inadequate drug levels due to non-adherence are most common cause of virologic failure without drug resistance.

The present study clearly indicates mandatory viral load testing for all ART experienced patients in regular basis. In addition to that prior to ART initiation, routine assessment of resistance associated mutations is extremely recommended. From India, evidence on virological failure without drug resistance mutations is very rare. This study will definitely help in better understanding the HIV drug resistance dynamics and planning in clinical management of HIV patients.

In chapter 5, we intended to define HIV diversity among babies born to HIV positive mother. Anti-retroviral drug resistance in pregnant women is one of the major factors for treatment failure in the patients with HIV. Efficacy of anti-retroviral regimen in preventing pre natal transmission may be hampered if the pregnant woman had the preexisting resistance to a drug in the ART regimen. The treatment for infant born to HIV positive mother is very limited if maternal drug resistance is transmitted to the fetus. HI drug resistance in pregnant women may be developed because of several factors. The adherence in AT may be compromised in

the early pregnancy as nausea and vomiting are usually experienced by all pregnant women. During pregnancy, pharmacokinetic changes i.e. increased in plasma volume and renal clearance may occur that lead to sub therapeutic drug resistance and risk of resistance may develop. Prevention of development of drug resistance to ARV regimen can be managed and maximum viral suppression can be achieved among pregnant women by regular monitoring of viral load and drug resistance testing and adherence to effective ARV regimen.

Predominant subtype C with some recombinant form has been found out in the present study that supports previously published data. No drug resistance mutation has been seen in the infants born to HIV positive mother. This data clearly indicates the effectiveness of ART to HIV positive pregnant mother. As per available data, nowadays the adherence to ART is increased and that results absence of HIV drug resistance in the present study.

All the above chapters urge for more emphasis on prevention, monitoring, detection and response to HIV drug resistance based on mandatory testing of viral load at regular intervals. Early detection of treatment failure in clinical, immunological and virologic parameter will assist to meet 95-95-95 targets sets by UNAIDS which ensure 95% people are tested for HIV, 95% people receiving HIV treatment, 95% of them achieve the HIV suppression by 2030 that lead to achieve Sustainable Development Goals.

Future Scope

The active changes in HIV-1 subtype diversity patterns pose global challenges for diagnosis, treatment and ARV drug design. Better characterization and extensive studies on CRFs are essential as new CRFs are increasing over time. Full length genome sequencing is essential to find out divergent HIV strains.

A successful rapid scale up of access to ART in India, warrants an urgent need of molecular HIV drug resistance surveillance.

For perinatally infected infants, diversity assay that detects drug resistance is highly recommended at the earliest to avoid unnecessary ARV drugs for the infants having preexisting resistance.

References

References

Abram ME, Ferris AL, Shao W, et al. Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. J Virol 2010; 84 (19): 9864-78

Adolescents PoAGfAa. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services 2014

Africa: HPTN 068. PLoS One 2018; 13: e0198999.

Aghokeng AF, Monleau M, Eymard-Duvernay S, et al. Virological outcome and frequency of drug resistance mutations in HIV-infected patients receiving first-line ARV regimen and monitored with the public health approach in Southeast Asia and sub-Saharan Africa. Antivir Ther. 2012;17:A122.

Andrade SD, Sabidó M, Monteiro WM, Benzaken AS, Tanuri A. Drug resistance in antiretroviral-naive children newly diagnosed with HIV-1 in Manaus, Amazonas. J Antimicrob Chemother. 2017 Jun 1;72(6):1774-1783.

Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D et al. (2008). "HIV-1 envelope protein binds to and signals through integrin alpha(4)beta(7), the gut mucosal homing receptor for peripheral T cells". *Nature Immunology*. In Press (3): 301–9.

Ashokkumar M, Aralaguppe SG, Tripathy SP, Hanna LE, Neogi U. Unique Phenotypic Characteristics of Recently Transmitted HIV-1 Subtype C Envelope Glycoprotein gp120: Use of CXCR6 Coreceptor by Transmitted Founder Viruses. J Virol. 2018 Apr 13;92(9):e00063-18.

Avanzi VM, Vicente BA, Beloto NCP, et al. Profile of HIV subtypes in HIV/HBV- and

Bakhouch K, Oulad-Lahcen A, Bensghir R, et al. The prevalence of resistance-associated mutations to protease and reverse transcriptase inhibitors in treatment-naive (HIV1)-infected individuals in Casablanca, Morocco. J Infect Dev Ctries 2009;3(5):380-91.

Balakrishnan P, Kumarasamy N, Kantor R, et al.: HIV type 1 genotypic variation in an antiretroviral treatment-naive population in southern India. AIDS Res Hum Retroviruses, 2005;21(4):301–305.

Balakrishnan P, Solomon S, Kumarasamy N, et al. Low-cost monitoring of HIV infected individuals on highly active antiretroviral therapy (HAART) in developing countries. Indian J Med Res 2005;121(4):345-55.

Barabona G, Mahiti M, Masoud S, Mbelele P, Mgunya AS, Minja L, Sunguya B, Shigemi U, Matsuda M, Hachiya A, Iwatani Y, Lyamuya E, Ueno T. Pre-treatment and acquired HIV drug resistance in Dar es Salaam, Tanzania in the era of tenofovir and routine viral load monitoring. J Antimicrob Chemother. 2019 Oct 1;74(10):3016-3020.

Basavapathruni A, Vingerhoets J, de Bethune MP, et al. Modulation of human immunodeficiency virus type 1 syn-ergistic inhibition by reverse transcriptase mutations. Biochemistry 2006; 45 (23): 7334-40

Becker-Pergola G, Kataaha P, Johnston-Dow L, et al.: Analysis of HIV type 1 protease and reverse transcriptase in antiretroviral drugnaive Ugandan adults. AIDS Res Hum Retroviruses 2000; 16: 807–813.

Bennett DE, Bertagnolio S, Sutherland D, et al. The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. Antivir Ther 2008;13(2):1-13.

Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, Kiuchi M et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PloS one. 2009;4(3): e4724.

Bhaskaran K, Hamouda O, Sannes M, Boufassa F, Johnson AM.Changes in the risk of death after HIV seroconversion compared with mortality in the general population. JAMA 2008;300: 51-59.

Bhatta, M., Dutta, N., Nandi, S., Dutta, S. and Saha, M.K., 2020. Mother-to-child HIV transmission and its correlates in India: systematic review and meta-analysis. *BMC pregnancy and childbirth*, *20*(1), pp.1-15.

Boden D, Hurley A, Zhang L, et al.: HIV-1 drug resistance in newly infected individuals. JAMA 1999; 282:1135–1141.

Burnett JC, Zaia JA, Rossi JJ. Creating genetic resistance to HIV. Curr Opin Immunol. 2012;24:625–632.

Buzon MJ, Codoner FM, Frost SD, Pou C, Puertas MC, Massanella M, et al. Deep molecular characterization of HIV-1 dynamics under suppressive HAART. PLoS Pathog. 2011;7

Cane P, Christie I, Dunn D, et al., UK Group on Transmitted HIV Drug Resistance: Time trends in primary resistance to HIV drugs in the United Kingdom: Multicentre observational study. BMJ 2005; 331:1368.

Chaix ML, Desquilbet L, Descamps D, et al. Response to HAART in French patients with resistant HIV-1 treated at primary infection: ANRS Resistance Network. Antivir Ther 2007; 12 (8): 1305-10

Chaudhuri S, Mundle M, Konar H, Das C, Talukdar A, Ghosh US. Utilization of therapeutic intervention to prevent mother to child transmission of HIV in a teaching hospital in Kolkata, India. J Obstet Gynaecol Res. 2010;36(3):619–25.

Chauhan CK, Lakshmi PVM, Sagar V, Sharma A, Arora SK, Kumar R. Primary HIV Drug Resistance among Recently Infected Cases of HIV in North-West India. AIDS Res Treat. 2019 Feb 27;2019:1525646.

Chen Z, Li Y, Schock HB, et al. Three-dimensional structure of a mutant HIV-1 protease displaying cross-resistance to all protease inhibitors in clinical trials. J Biol Chem 1995;270(37):21433-6.

Clinton lauds India Aids campaign". 26 May 2005. Retrieved 6 April 2018 – via news.bbc.co.uk.

Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 1995; 267 (5197): 483-9

Condra JH, Holder DJ, Schleif WA, et al. Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J Virol 1996;70(12):8270-6.

Cornelissen M, Van DB, Zorgdrager F, et al.: pol gene diversity of five human immunodeficiency virus type 1 subtypes: evidence for naturally occurring mutations that contribute to drug resistance, limited recombination patterns, and common ancestry for subtypes B and D. J Virol 1997; 71:6348–6358.

Damania KR, Tank PD. Prevention of mother to child transmission of HIV infection. J Obstet Gynecol India. 2006;56(5):390–5.

De Cock KM, Fowler MG, Mercier E, et al. Prevention of mother-to child HIV transmission in resource-poor countries: translating research into policy and practice. *JAMA* 2000; 283:1175–1182.

Deeks SG. HIV infection, inflammation, immunosenescence, and aging. Annu Rev Med. 2011;62:141–155.

Desai M, Iyer G, Dikshit RK. Advances in antiretroviral drugs. Indian J Pharmacol. 2012;44:288-98.

Deshpande A, Jauvin V, Magnin N, et al.: Resistance mutations in subtype C HIV type 1 isolates from Indian patients of Mumbai receiving NRTIs plus NNRTIs and experiencing a treatment failure: Resistance to AR. AIDS Res Hum Retroviruses 2007;23(2):335–340.

Deshpande A, Recordon-Pinson P, Deshmukh R. Molecular characterization of HIV type 1 isolates from untreated patients of Mumbai (Bombay), India and detection of rare resistance mutations. AIDS Res Hum Retroviruses 2004; 20: 1032-35.

Dhadwal D, Bhardwaj A, Gupta A, Sharma S, Parashar A, Thakur A, Mahajan A, Chander V, Sood A. Role of intensive training in strengthening the skills of HIV counselors for imparting quality ICTC services. Indian J Community Med. 2009;34(3):252–4.

Di Giallonardo F, Pinto AN, Keen P, Shaik A, Carrera A, Salem H, Selvey C, Nigro SJ, Fraser N, Price K, Holden J, Lee FJ, Dwyer DE, Bavinton BR, Grulich AE, Kelleher AD, On Behalf Of The Nsw Hiv Prevention Partnership Project. Increased HIV Subtype Diversity Reflecting Demographic Changes in the HIV Epidemic in New South Wales, Australia. Viruses. 2020 Dec 6;12(12):1402.

Doepker LE, Simonich CA, Ralph D, Shipley MM, Garrett M, Gobillot T, Vigdorovich V, Sather DN, Nduati R, Matsen FA 4th, Overbaugh JM. Diversity and Function of Maternal HIV-1-Specific Antibodies at the Time of Vertical Transmission. J Virol. 2020 Apr 16;94(9):e01594-19.

Douek DC, Roederer M, Koup RA (2009). "Emerging Concepts in the Immunopathogenesis of AIDS". *Annu. Rev. Med.* 60: 471–84.

Dreja H et. al. Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection. J Gen Virol 2010; 91:2794-2803.

Duani H, Aleixo AW, Tupinambás U. Trends and predictors of HIV-1 acquired drug resistance in Minas Gerais, Brazil: 2002-2012. Braz J Infect Dis. 2017 Mar-Apr;21(2):148-154

Dulude D, Théberge-Julien G, Brakier-Gingras L, Heveker N. Selection of peptides interfering with a ribosomal frameshift in the human immunodeficiency virus type 1. RNA. 2008 May;14(5):981-91.

Dutta N, Nandi S, Guha S, et al. Emergence of HIV drug-resistant mutations in East Indian population after failure of first-line antiretroviral therapy. HIV and AIDS Review. International Journal of HIV-Related Problems 2017;16(4):258-64.

Dutta N, Omesh S, Nandi S, et al. Virologic failure on anti-retroviraltherapy without HIV drug resistance mutation. J Immune Disord Ther2018;1(1):1-5.

Easterbrook PJ, Smith M et al. Impact of HIV-1 subtype on disease progression and response to antiretroviral therapy. J. Int AIDS Soc 2010; 13:4.

Emlet CA., 2006. A comparison of HIV stigma and disclosure patterns between older and younger adults living with HIV/AIDS. AIDS Patient Care STDS. 20:350–358.

Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. Nat Rev Microbiol. 2012 Mar 16;10(4):279-90. doi: 10.1038/nrmicro2747. PMID: 22421880; PMCID: PMC3588166.

Eshleman H, Hackett J, Swanson .Performance of the Celera Diagnostics ViroSeq HIV-1 genotyping system for sequence-based analysis of diverse human immunodeficiency virus type 1 strains. J. Clin. Microbiol 2004;42:2711–2717.

Eshleman SH, Hudelson SE, Gupta A, et al.: Limited evolution in the HIV type 1 pol region among acute seroconverters in Pune, India. AIDS Res Hum Retroviruses 2005;21(1): 93–97.

Fanales-Belasio, E., Raimondo, M., Suligoi, B. and Buttò, S., 2010. HIV virology and pathogenetic mechanisms of infection: a brief overview. Annali dell'Istituto superiore di sanita, 46, pp.5-14.

Faria NR, Rambaut A, Suchard MA, Baele G, Bedford T, Ward MJ et al. (2014). "The early spread and epidemic ignition of HIV-1 in human populations".*Science* 346 (6205): 56–61.

Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997;278:1295–1300.

Frentz D, Boucher CA, van de Vijver DA. Temporal changes in the epidemiology of transmission of drug-resistant HIV-1 across the world. AIDS Rev. 2012;14(1):17–27.

Gadkari DA, Moore D, Sheppard HW, Kulkarni SS. Transmission of genetically diverse strains of HIV-1 in Pune, India. Indian J Med Res 1998; 107: 1-9.

Ganser-Pornillos BK, Yeager M, Sundquist WI. The structural biology of HIV assembly. Curr Opin Struct Biol. 2008 Apr;18(2):203-17.

Gatell, J.M., Morales-Ramirez, J.O., Hagins, D.P., Thompson, M., Keikawus, A., Hoffmann, C., Rugina, S., Osiyemi, O., Escoriu, S., Dretler, R. and Harvey, C., 2014. Forty-eight-week efficacy and safety and early CNS tolerability of doravirine (MK-1439), a novel NNRTI, with TDF/FTC in ART-naive HIV-positive patients. Journal of the International AIDS Society, 17(4S3).

Geretti, Anna Maria. HIV-1 subtypes: epidemiology and significance for HIV management. Current Opinion in Infectious Diseases, February 2006- Volume 19- Issue 1- p1-7

Gilbert PB, McKeague IW, Eisen G, Mullins C, Guéye-NDiaye A, Mboup S et al. (February 28, 2003). "Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal". *Statistics in Medicine* 22 (4): 573–593

Girón-Callejas A, García-Morales C, Mendizabal-Burastero R, Meza RI, Sierra T, Tapia-Trejo D, Pérez-García M, Quiroz-Morales VS, Paredes M, Rodríguez A, Juárez SI, Farach N, Videa G, Lara B, Rodríguez E, Ardón E, Sajquim E, Lorenzana R, Ravasi G, Northbrook S, Reyes-Terán G, Ávila-Ríos S. High level of pre-treatment and acquired HIV drug resistance in Honduras: a nationally representative survey, 2016-17. J Antimicrob Chemother. 2020 Jul 1;75(7):1932-1942

Global update on HIV treatment 2013: results, impact and opportunities. WHO report in partnership with UNICEF and UNAIDS; 2013.

Goonetilleke N, Liu MK, Salazar-Gonzalez JF, et al. The first T cell response to transmitted/founder virus con-tributes to the control of acute viremia in HIV-1 infection. J Exp Med 2009; 206 (6): 1253-72

Goudsmit, Jaap. Viral Sex; The Nature of AIDS. Oxford University Press. New York, New York, 1997. Pg. 51-58. Retrieved May 25, 2008.

Gounder K, Oyaro M, Padayachi N, et al. Complex subtype diversity of HIV-1 among drug user in major Kenyan cities. AIDS Res Hum Retroviruses 2017; 33:500–510.A study that highlights extensive HIV-1 inter-subtype genetic diversity in East Africa and changing dynamics in the prevalence of pure viral subtypes.

Gupta A, Saple DG, Nadkarni G, et al. One-, two-, and three-class resistance among HIV-infected patients on antiretroviral therapy in private care clinics: Mumbai, India. AIDS Res Hum Retroviruses 2010;26:25-31.

Gupta R, Hill A, Sawyer AW, et al. Emergence of drug resistance in HIV type 1-infected patients after receipt of first-line highly active antiretroviral therapy: a systematic review of clinical trials. Clin Infect Dis 2008; 47 (5): 712-22

Haile GS, Berha AB. Predictors of treatment failure, time to switch and reasons for switching to second line antiretroviral therapy in HIV infected children receiving first line anti-retroviral therapy at a Tertiary Care Hospital in Ethiopia. BMC Pediatr. 2019 Jan 29;19(1):37.

Hamers R, Wallis CL, Kityo C, Siwale M, Mandaliya K. HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy: a multicentre observational study. Lancet Infect Dis 2011;11: 750-759.

Hemelaar J, Gouws E, et. al., Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS 2011; 25: 679-689.

Herbeck JT, Muller V, et al. Is the virulence of HIV changing? A meta-analysis of trends in prognostic markers of HIV disease progression and transmission. AIDS2012; 26:193–205.

Hertogs K, Bloor S, Kemp SD, et al. Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples. AIDS 2000;14:1203-10.

Hill A et al. Resistance at virological failure using boosted protease inhibitors versus nonnucleoside reverse transcriptase inhibitors as first-line antiretroviral therapy--implications for sustained efficacy of ART in resource-limited settings. 2013 J Infect Dis. Jun 15;207 Suppl 2:S78-84.

Hira SK, Panchal K, Parmar PA, Bhatia VP. High resistance to antiretroviral drugs: the Indian experience. Int J STD AIDS 2004; 15: 173-77.

HIV Sentinel Surveillance 2012-13: A Technical Brief, *National AIDS Control Organization*, New Delhi.

HIV/HCV-coinfected patients in Southern Brazil. Rev Soc Bras Med Trop 2017; 50:470–477.

Hompe ED, Mangold JF, Kumar A, Eudailey JA, McGuire E, Haynes BF, Moody MA, Wright PF, Fouda GG, Giorgi EE, Gao F, Permar SR. Induction of Neutralizing Responses against Autologous Virus in Maternal HIV Vaccine Trials. mSphere. 2020 Jun 3;5(3):e00254-20.

Hosseinipour MC, van Oosterhout JJ, Weigel R, Phiri S, Kamwendo D. The public health approach to identify antiretroviral therapy failure: high-level nucleoside reverse

transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy. AIDS 2009;23: 1127-1134.

http://www.unaids.org/sites/default/files/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf

https://www.unaids.org/en/resources/fact-sheet, 2021

Hurt, Christopher B. "Transmitted Resistance to HIV Integrase Strand-Transfer Inhibitors: Right on Schedule." *Antiviral therapy* 16.2 (2011): 137–140. *PMC*. Web. 22 Apr. 2015.

Hwan Bang JI, Song KH, Kim S-H, et al.: Prevalence of primary antiretroviral resistance: Trends in Korea. AIDS Res Hum Retroviruses 2008; 24(1):83–85.

Ibe S, Hattori J, Fujisaki S, et al.: Tend of drug resistant HIV Type 1 emergence among therapy-naive patients in Nagoya Japan: An 8-year surveillance from 1999 to 2006. AIDS Res Hum Retroviruses 2008;24(1):7–14.

India: Driving forward an effective AIDS response. unaids.org. Retrieved 6 April 2018

infection. J Obstet Gynecol India. 2006;56(5):390–5.

International Committee on Taxonomyof Viruses (2002). "61.06. Lentivirus". National Institute of Health. Retrieved Frbruary 28, 2006.

Irene YV, Arun A. Efficacy of single dose nevirapine in prevention of mother to child transmission of HIV-1. J Obstet Gynecol India. 2010;60(3):221–4

Ives NJ, Gazzard BG, Easterbrook PJ. The changing pattern of AIDS-defining illnesses with the introduction of highly active antiretroviral therapy (HAART)in a London clinic. J Infect 2001; 42: 134-139.

Jahn A, Floyd S, Crampin AC, Mwaungulu F, Mvula H.Population-level effect of HIV on adult mortality and early evidence of reversal after introduction of antiretroviral therapy in Malawi. Lancet 2008; 371: 1603-1611.

Jameel, S., M. Zafrullah, et. al. 1995. A genetic analysis of HIV-1 from Punjab, India reveals the presence of multiple variants. AIDS 9:685–690.

Joint UN Programme on HIV/AIDS and World Health Organization. AIDS epidemic update: World Health Organization, Geneva, Switzerland, December 2007

Kaleebu P, Ross A et. al. Relationship between HIV-1 Env subtypes A and D and Disease progression in a rural Ugandan cohort. AIDS 2001; 15: 293-299.

Kamenju P, Liu E, Hertzmark E, Spiegelman D, Kisenge R, Kupka R, Aboud S, Manji KP, Duggan C, Fawzi WW. Nutritional status and complementary feeding among HIV-exposed infants: a prospective cohort study. Matern Child Nutr. 2017 Jul;13(3):e12358.

Kandathil AJ, Kannangai R, Verghese VP, et al. Drug resistant mutations detected by genotypic drug resistance testing in patients failing therapy in clade C HIV-1 infected individuals from India. Indian J Med Microbiol 2009;27(3):231-6.

Kantor R, Diero L, et al. Misclassification of first-line antiretroviral treatment failure based on immunological monitoring of HIV infection in resource-limited settings. Clin InfectDis. 2009;49(3):454_62.

Kantor R, Katzenstein D. Drug resistance in non-subtype B HIV-1. J Clin Virol 2004; 29: 152-59.

Kantor R. et. al. HIV diversity and drug resistance from plasma and non-plasma analytes in a large treatment programme in western Kenya. Journal of the International AIDS Society 2014, 17:19262.

Kearney MF, et al. Lack of detectable HIV-1 molecular evolution during suppressive antiretroviral therapy. PLoS Pathog. 2014;10(3):e1004010.

Kerina, Duri et al. “HIV Diversity and Classification, Role in Transmission.” Advances in Infectious Diseases 03 (2013): 146-156

Khan IF, Vajpayee M, Prasad VSP, Seth P. Genetic diversity of HIV type 1 subtype C env gene from India. AIDS Res Hum Retroviruses 2007; 23: 934-40.

KLole S. K, Robert C, Bollinger, Paranjape R. S,Gadkari D, Kulkarni S. S, Nicole G, Novak, Ingersoll R, Haynes W, Sheppard and Roy Stuart C: Full -Length Human Immunodeficiency Virus Type1 Genomes from Subtype C-Infected Seroconverters in

India, with Evidence of Intersubtype Recombination. Journal of Virology, Jan 1999; Vol. 73, p 152-160

Korber B, Gaschen B, et al. Evolutionary and immunological implications of contemporary HIV-1 variation. Br Med Bull 2001; 58: 19-42

Kozal M. Cross-resistance patterns among HIV protease inhibitors. AIDS Patient Care STDs. 2004;18(4):199-208.

Kulkarni S. S., Lapedes A. et al. Highly complex neutralization determinants on a monophyletic lineage of newly transmitted subtype C HIV-1 Env clones from India. Virology 15 March 2009, olume 385, Issue 2, Pages 505–520.

Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. Brief Bioinform 2004; 5: 150-63.

Laboratory Guidelines for HIV Diagnosis in Infants and Children less than 18 months. Ministry of Health & Family Welfare, NACO, New Delhi January 2011.

Lakshmi R, Ramesh K, Hanna LE, Narayanan PR, Swaminathan S. Emergence of drug resistant mutations after single dose nevirapine exposure in HIV-1 infected pregnant women in south India. Indian J Med Res 2010; 132: 509-12.

Lakshmi R, Ramesh K, Narayanan PR, Swaminathan S. Antiretroviral drug-resistant mutations at baseline and at time of failure of antiretroviral therapy in HIV type 1-coinfected TB patients. AIDS Res Hum Retroviruses 2009; 25: 1179-85.

Larder BA. Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse tran-scriptase. J Gen Virol 1994; 75: 951-7

Le Rouzic E, Benichou S. The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology. 2005 Feb 22;2:11.

Leger P, Charles M, Severe P, Riviere C, Pape JW. 5-year survival of patients with AIDS receiving antiretroviral therapy in Haiti. N Engl J Med 361: 828-829;2009.

Levy JA. (2007). HIV and the Pathogenesis of AIDS. 3rd ed.Washington: ASM Press.

Levy JA. HIV and pathogenesis of AIDS. 3rd ed. Washington: ASM Press; 2007

Little SJ, Holte S, Routy JP, et al. Antiretroviral-drug resistance among patients recently infected with HIV (2002) New Engl J Med, 2002;347(6):385–394.

Lodi S, Günthard HF, Dunn D, Garcia F, Logan R, Jose S, Bucher HC, Scherrer AU, Schneider MP, Egger M, Glass TR, Reiss P, van Sighem A, Boender TS, Phillips AN, Porter K, Hawkins D, Moreno S, Monge S, Paraskevis D, Simeon M, Vourli G, Sabin C, Hernán MA; HIV-CAUSAL Collaboration. Effect of immediate initiation of antiretroviral treatment on the risk of acquired HIV drug resistance. AIDS. 2018 Jan 28;32(3):327-335

Mandal S, Bhattacharya RN, Chakraborty M, Pal PP, Roy SG, Mukherjee G. Evaluation of the prevention of parent to child transmission program in a rural tertiary care hospital of West Bengal, India. Indian J Community Med. 2010;35(4):491.

Manosuthi W, Thongyen S, Nilkamhang S, et al. HIV-1 drug resistance-associated mutations among antiretroviral-naive Thai patients with chronic HIV-1 infection. J Med Virol 2013;85(2):194-9.

Mansky LM. HIV mutagenesis and the evolution of anti-retroviral drug resistance. Drug Resist Updat 2002; 5 (6): 219-23

Marc A. et al. Luc Montagnier- Discoverer of the AIDS Virus. Stamp Vignette on Medical Science. Vol. 77, Issue 6, P506, June 01, 2002. DOI:https://doi.org/10.4065/77.6.506

Marcello A, Lusic M, Pegoraro G, Pellegrini V, Beltram F, Giacca M. Nuclear organization and the control of HIV-1 transcription. Gene. 2004 Feb 4;326:1-11.

Me´decins Sans Frontie`res (MSF) (2013) Access campaign. Untangling the web of antiretroviral price reductions. 16th edn. Geneva, Switzerland: Me´decins Sans Frontie`res.

Michele W. et al., HIV-1 Antiretroviral Resistance Scientific Principles and Clinical Applications. Drugs 2012; 72 (9): e1-e25.

Miller V. Resistance to protease inhibitors. J Acquir Immune Defic Syndr 2001;26:S34-50.

Mills EJ, Bakanda C, Birungi J, Chan K, Ford N.Life expectancy of persons receiving combination antiretroviral therapy in low-income countries: a cohort analysis from Uganda. Ann Intern Med 2011; 155: 209-216.

Ministry of Health and Family Welfare, Government of India 2011. NACO. National guidelines on second line ART.

Msimanga P. W. et. al. HIV-1 diversity in an antiretroviral treatment naïve cohort from Bushbuckridge, Mpumalanga Province, South Africa. Virology Journal 2015 12:24.

NACO, HIV Testing manual: Laboratory Diagnosis, Biosafety and Quality Control.www.nacoonline.org.

NACO, HIV Testing policy and functioning of VCTC.www.nacoonline.org.

NACO. Annual technical report: India HIV estimations 2017.

NACO. Guidelines for the prevention of mother to child transmission of HIV. Available from: http://www.nacoonline.org/guidelines/guideline_9.pdf

NACO. HIV Testing manual: Laboratory Diagnosis, Biosafety and Quality Control.

NACO. HIV Testing policy and functioning of VCTC.

NACO. Updated guidelines for prevention of parent to child transmission (PPTCT) of HIV using multi drug anti-retroviral regimen in India, 2013.

Nandi. S, S Maity, S C Bhunia, M K Saha. 2014. Comparative assessment of commercial ELISA kits for detection of HIV in India. BMC Res Notes 7:436. doi:10.1186/1756-0500-7-436.

Nankya I, Mehta S, Akao J, et al. Trends of HIV-1 drug resistance during the past 11 years of ARV treatment in Uganda. Antivir Ther. 2012;17:A127.

Nath A. Pediatric HIV in India: current scenario and the way forward. Indian J Public Health. 2017;61(2):124.

National AIDS Control Organization & ICMR-National Institute of Medical Statistics (2022). India HIV Estimates 2021: Fact Sheet. New Delhi: NACO, Ministry of Health and Family Welfare, Government of India.

Ndung'u T, Weiss RA. On HIV diversity. *AIDS. 2012* Jun 19;26(10):1255-60

Novak RM, Chen L, Mac Arthur RD, et al.: Prevalence of antiretroviral drug resistance mutations in chronically HIV-infected treatment naive patients: Implications for routine resistance screening before initiation of antiretroviral therapy. Clin Infect Dis 2005; 40(3):468–474.

Novak RM, Chen L, MacArthur RD, et al. Prevalence of antire- troviral drug resistance mutations in chronically HIV-infected, treatment-naive patients: implications for routine resistance screening before initiation of antiretroviral therapy. Clin Infect Dis 2005;40(3):468-74.

Orrell C, Walensky RP, Losina E, et al. HIV type-1 clade C resistance genotypes in treatment-naive patients and after first virological failure in a large community antiretroviral therapy programme. Antivir Ther 2009;14(4):523-31.

Palma P, Zangari P, Alteri C, Tchidjou HK, Manno EC, Liuzzi G, Perno CF, Rossi P, Bertoli A, Bernardi S. Early antiretroviral treatment (eART) limits viral diversity over time in a long-term HIV viral suppressed perinatally infected child. BMC Infect Dis. 2016 Dec 9;16(1):742.

Pao D, Andrady U, Clarke J, et al. Long-term persistence of primary genotypic resistance after HIV-1 seroconver-sion. J Acquir Immune Defic Syndr 2004; 37 (5): 1570-3

Paranjpe, S.M., Sarkate, P.P., Ingole, N.A., Raut, S.S. and Mehta, P.R., 2016. Profiles of HIV-infected anti-retroviral therapy naïve children from Mumbai, India. World Journal of Pediatrics, 12(4), pp.430-435.

Pepin J. The Origin of AIDS. Cambridge, UK: Cambridge University Press; 2011.

Perelson AS, Neumann AU, Markowitz M, et al. HIV-1 dynamics in vivo: Virion clearance rate, infected cell life-span, and viral generation time. Science 1996; 271 (5255): 1582-6

Perelson AS, Ribeiro RM (October 2008). "Estimating drug efficacy and viral dynamic parameters: HIV and HCV". *Stat Med* 27 (23): 4647–57.

Pieniazek D, Rayfield M, Hu DJ, et al.: Protease sequences from HIV-1 group M subtypes A–H reveal distinct amino acid mutation patterns associated with protease resistance in protease inhibitornaive individuals worldwide. HIV Variant Working Group. AIDS 2000; 14:1489–1495.

Radhakrishnan P. India: The Department for International Development; 2007. India and second line ART: evaluating the way forward.

Rapheal W. Lihana et al. Update on HIV-1 Diversity in Africa: A decade in Review. AIDS Rev. 2012; 14:83-100

Ray, S. C. 1998. SimPlot for Windows (version 1.6). Baltimore, Md. Distributed by author (http://www.welch.jhu.edu/;sray/download).

Re MC, Monari P, Bon I, et al. Analysis of HIV-1 drug resistant mutations by line probe assay and direct sequencing in a cohort of therapy naive HIV-1 infected Italian patients. BMC Microbiol 2001;1:30.

Re MC, Monari P, Bon I, et al. Conflicting interpretations of the prevalence of mutations associated with drug resistance in antiviral naive HIV-1 patients with acute and chronic infection. Int J Antimicrob Agents 2004;23(2):164-8.

Reynolds SJ, Ssempijja V, Galiwango R, Ndyanabo A, Nakigozi G, Lyagoba F, Nazziwa J, Redd A, Lamers SL, Gray R, Wawer M, Serwadda D, Quinn TC. Low Rates of Transmitted Drug Resistance Among Newly Identified HIV-1 Seroconverters in Rural Rakai, Uganda. AIDS Res Hum Retroviruses. 2017 May;33(5):448-451.

Robertson DL, Anderson JP, et al. HIV-1 nomenclature proposal. Science 2000; 288: 55-56.

Rose KM, Marin M, Kozak SL, Kabat D. The viral infectivity factor (Vif) of HIV-1 unveiled. Trends Mol Med. 2004 Jun;10(6):291-7.

Rubio-Garrido M, González-Alba JM, Reina G, Ndarabu A, Barquín D, Carlos S, Galán JC, Holguín Á. Current and historic HIV-1 molecular epidemiology in paediatric and

adult population from Kinshasa in the Democratic Republic of Congo. Sci Rep. 2020 Oct 28;10(1):18461.

S Schwartz, B K Felber, D M Benko, E M Fenyö, and G N Pavlakis (1990). Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol.64 (6): 2519-29

Saha M, Bhattacharya S. A Brief Overview on HIV Infection, Diagnosis and Treatment. Curr Top Med Chem. 2019;19(30):2739-2741. doi: 10.2174/1568026619302001030913335. PMID: 31908209.

Sahni AK. et. al . Genomic diversity of human immunodeficiency virus type-1 in India. Int J STD AIDS 2002 Feb;13(2):115-8

Schwartländer B, Stover J, Hallett T, Atun R, Avila C. Towards an improved investment approach for an effective response to HIV/AIDS. Lancet 2011; 377: 2031-2041.

Segujja F, Omooja J, Lunkuse S, Nanyonjo M, Nabirye SE, Nassolo F, Bugembe DL, Bbosa N, Kateete DP, Ssenyonga W, Mayanja Y, Nsubuga RN, Seeley J, Kaleebu P, Ssemwanga D. High Levels of Acquired HIV Drug Resistance Following Virological Nonsuppression in HIV-Infected Women from a High-Risk Cohort in Uganda. AIDS Res Hum Retroviruses. 2020 Sep;36(9):782-791.

Sen S, Tripathy SP, Chimanpure VM, Patil AA, Bagul RD, and Paranjape RS: Human immunodeficiency virus type 1 drug resistance mutations in peripheral blood mononuclear cell proviral DNA among antiretroviral treatment-naive and treatment-experienced patients from Pune, India. AIDS Res Hum Retroviruses 2007;23 (4):489–497.

Sen S, Tripathy SP, Patil A, Chimanpure VM, and Paranjape RS: High prevalence of HIV-1 drug resistance mutation in antiretroviral treatment experienced patients from Pune, India. AIDS Res Hum Retroviruses 2007;23(10):1303–1308.

Shafer RW, Rhee SY, Bennett DE. Consensus drug resistance mutations for epidemiological surveillance: basic principles and potential controversies. Antivir Ther. 2008;13 Suppl 2:59–68.

Shankarappa R., Chatterjee R. et al. Human Immunodeficiency Virus Type 1 Env Sequences from Calcutta in Eastern India: Identification of Features That Distinguish Subtype C Sequences in India from Other Subtype C Sequences. Journal of Virology Nov. 2001, p. 10479–10487.

Sharma AL, Singh TR, Devi KR, Singh LS. Molecular epidemiology of HIV-1 among the HIV infected people of Manipur, North-eastern India: emergence of unique recombinant forms. J Med Virol 2017; 89:989–999.

Sharp PM, Hahn BH (2011). "Origins of HIV and the AIDS Pandemic". *Cold Spring Harbor perspectives in medicine* 1 (1): a006841

Sharp PM, Hahn BH et al. The evolution of HIV-1 and the origin of AIDS. Philos Trans R Soc Lond B Biol Sci 2010; 365:2487-2494

Sharp, P.M. & Hahn, B.H. (2011) 'Origins of HIV and the AIDS pandemic' Cold Spring Harbour Perspectives in Medicine 1(1):a006841

Sinha A, Roy M. An ICMR task force study of prevention of parent to child transmission (PPTCT) service delivery in India. Indian J Public Health. 2008; 52(4):200–2.

Sinha S, Ahmad H, Shekhar RC, et al. Prevalence of HIV Drug Resistance Mutations in HIV Type 1 Isolates in Antiretroviral Therapy Naïve Population from Northern India. *AIDS Res Treat.* 2012; 2012:905823. Epub 2012 Mar 15.

Sivay MV, Hudelson SE, Wang J, et al. HIV-1 diversity among young women in rural South

Smith DM, Schooley RT. Running with scissors: using antiretroviral therapy without monitoring viral load. Clin Infect Dis 2008;46(10): 1598-600.

Soundararajan L, Karunaianandham R, Jauvin V, et al.: Characterization of HIV-1 isolates from antiretroviral drug-naive children in southern India. AIDS Res Hum Retroviruses 2007;23(9):1119–1126.

Stanford University HIV Drug Resistance Database IVDR), http://hivdb.stanford.edu.

Stanford University HIV Drug Resistance Database IVDR.

Suryavanshi N, Mave V, Kadam A, Kanade S, Sivalenka S, Kumar VS, Harvey P, Gupta R, Hegde A, Gupte N, Gupta A. Challenges and opportunities for outreach workers in the prevention of mother to child transmission of HIV (PMTCT) program in India. PLoS One. 2018;13(9):e0203425

Sutcliffe CG, van Dijk JH, Muleka M, Munsanje J, Thuma PE, Moss WJ. Delays in Initiation of Antiretroviral Therapy Among HIV-infected Children in Rural Zambia. Pediatr Infect Dis J. 2016 Apr;35(4):e107-12.

Tamura K. and Nei M. (**1993**). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**:512-526.

Tamura K., Battistuzzi FU, Billing-Ross P, Murillo O, Filipski A, and Kumar S. (**2012**). Estimating Divergence Times in Large Molecular Phylogenies. *Proceedings of the National Academy of Sciences* 109:19333-19338.

Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. (**2013**). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*30: 2725-2729.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence weighting, position specific alignment through sequence weight matrix choice. Nucleic Acids Res 1994; 22: 4673-80.

Thompson MA, Aberg JA, Hoy JF, et al. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. JAMA. 2012;308(4):387-402.

Thomson MM, Pérez-Alvarez L, Nájera R (2002). "Molecular epidemiology of HIV-1 genetic forms and its significance for vaccine development and therapy".*Lancet Infectious Diseases* 2 (8): 461–471.

Toni T, Masquelier B, Minga A, et al. HIV-1 antiretroviral drug resistance in recently infected patients in Abidjan, Cote d'Ivoire: A 4- year survey, 2002-2006. AIDS Res Hum Retroviruses 2007;23(9): 1155-60.

UNAIDS, 2011 UNAIDS World AIDS Day Report; 2011.

UNAIDS. On the fast-track to end AIDS: UNAIDS 2016–2021 Strategy. http://www.unaids.org/sites/default/files/media_asset/20151027_UNAIDS_PCB37_15_18_EN_rev1.pdf

UNAIDS.A progress report on the Global Plan towards the elimination of new HIV infections among children by 2015 and keeping their mothers alive;2012.

UNAIDS: the Gap Report, Geneva, 2014 http://www.unaids.org/en/resources/documents/2014/name,97466,en.asp

UNAIDS; WHO (December 2007). "2007 AIDS epidemic update" (PDF). p. 10. Retrieved 2008-03-12.

United Nations. United Nations High Level meeting on AIDS;2011.

Use of antiretroviral drugs for treating pregnant women and preventing HIV infection in infants. Rapid Advice, WHO guidelines, World Health Organization, Geneva, Switzerland. November, 2009.

Van Zyl GU, Katusiime MG, Wiegand A, McManus WR, Bale MJ, Halvas EK, Luke B, Boltz VF, Spindler J, Laughton B, Engelbrecht S, Coffin JM, Cotton MF, Shao W, Mellors JW, Kearney MF. No evidence of HIV replication in children on antiretroviral therapy. J Clin Invest. 2017 Oct 2;127(10):3827-3834.

Vandamme AM, Camacho RJ, Ceccherini-Silberstein F, et al. European recommendations for the clinical use of HIV drug resistance testing: 2011 update. AIDS Rev 2011;13(2):77-108.

Various (2008). HIV Sequence Compendium 2008 Introduction (PDF). Retrieved March 31, 2009.

Vella S, Schwartlander B et al. The history of Antiretroviral therapyand its implementation in resource limited areas of the world. AIDS 2012; 26:1231-1241

Vidya M, Saravanan S, Uma S, et al. Genotypic HIV type-1 drug resistance among patients with immunological failure to first-line antiretroviral therapy in south India. Antivir Ther 2009;14(7):1005-9.

Votteler J, Schubert U. Ubiquitin ligases as therapeutic targets in HIV-1 infection. Expert Opin Ther Targets. 2008 Feb;12(2):131-43.

Waheed AA, Freed EO. Lipids and membrane microdomains in HIV-1 replication. Virus Res. 2009 Aug;143(2):162-76

Walmsley S, Bernstein B, King M, et al. Lopinavir-ritonavir versus nelfinavir for the initial treatment of HIV infection. N Engl J Med 2002;346(26):2039-46.

Weidle PJ, Downing R, Sozi C, et al.: Development of phenotypic and genotypic resistance to antiretroviral therapy in the UNAIDS HIV Drug Access Initiative-Uganda. AIDS 2003;17 (Suppl 3): S39–S48.

Weiss RA (May 1993). "How does HIV cause AIDS?" *Science* 260 (5112): 1273–9.

Wensing AM, van de Vijver DA, Angarano G, et al. Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. J Infect Dis 2005;192(6): 958-66.

Whitcomb JM, Huang W, Limoli K, et al. Hypersuscept-ibility to non-nucleoside reverse transcriptase inhibitors in HIV-1: clinical, phenotypic and genotypic correlates. Aids 2002; 16 (15): F41-7

WHO, UNAIDS, UNICEF. Global HIV/AIDS response: epidemic update and health sector progress towards universal access; 2015.

WHO, UNAIDS, UNICEF. Global HIV/AIDS response: epidemic update and health sector progress towards universal access; 2011.

WHO. Global guidance on criteria and processess for validation: elimination of mother-to-child transmission of HIV and syphilis. 2nd ed; 2017

Wittkop L, Gunthard HF, de Wolf F, et al. Effect of transmitted drug resistance on virological and immuno-logical response to initial combination antiretroviral therapy for HIV

(EuroCoord-CHAIN joint project): a European multicohort study. Lancet Infect Dis 2011; 11 (5): 363-71.

Wolff M, Shepherd BE, Cortés C, Rebeiro P, Cesar C, Wagner Cardoso S, Pape JW, Padgett D, Sierra-Madero J, Echevarria J, McGowan CC; Caribbean, Central and South America Network for HIV Epidemiology. Clinical and Virologic Outcomes After Changes in First Antiretroviral Regimen at 7 Sites in the Caribbean, Central and South America Network. J Acquir Immune Defic Syndr. 2016 Jan 1;71(1):102-10.

Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. 1997;278:1291–1295.

World Health Organization. HIV drug resistance report 2017. http://www.who.int/hiv/pub/drugresistance/hivdr-report-2017/en/ (Accessed 30 December 2018). 2017.

World Health Organization. PMTCT strategic vision 2010–2015: preventing mother-to-child transmission of HIV to reach the UNGASS and Millennium Development Goals; 2010.

Worobey, M. et al (2010) 'Island biogeography reveals the deep history of SIV' Science 329(5998):1487

Xu, X., Luo, L., Song, C., Li, J., Chen, H., Zhu, Q., Lan, G., Liang, S., Shen, Z., Cao, Z. and Feng, Y., 2021. Survey of pretreatment HIV drug resistance and the genetic transmission networks among HIV-positive individuals in southwestern China, 2014–2020. BMC infectious diseases, 21(1), pp.1-10

Yazdanpanah Y, Fagard C, Descamps D, et al. High rate of virologic suppression with raltegravir plus etravirine and darunavir/ritonavir among treatment-experienced patients infected with multidrug- resistant HIV: results of the ANRS 139 TRIO trial. Clin Infect Dis. 2009;49(9):1441-9.

Yuan D, Liu M, Jia P, Li Y, Huang Y, Ye L, Api L, Chen M, Yao L, Wang Z, Liu H, Liang S, Yang S. Prevalence and determinants of virological failure, genetic diversity and

drug resistance among people living with HIV in a minority area in China: a population-based study. BMC Infect Dis. 2020 Jun 23;20(1):443.

Yuan H, Liu Z, Wu X, Wu M, Fang Q, Zhang X, Shi T, Tully DC, Zhang T. Prevalence of transmitted HIV-1 drug resistance among treatment-naive individuals in China, 2000-2016. Arch Virol. 2021

Yukl SA, Shergill AK, McQuaid K, Gianella S, Lampiris H, Hare CB, et al. Effect of raltegravir-containing intensification on HIV burden and T-cell activation in multiple gut sites of HIV-positive adults on suppressive antiretroviral therapy. AIDS. 2010;24:2451–2460.

Zeh C, Weidle PJ, Nafisa L, Lwamba HM, Okonji J, et al. (2011) HIV-1 Drug Resistance Emergence among Breastfeeding Infants Born to HIV-Infected Mothers during a Single-Arm Trial of Triple-Antiretroviral Prophylaxis for Prevention of Mother-To-Child Transmission: A Secondary Analysis. PLOS Medicine 8(3): e1000430.

Zhang F, Dou Z, Ma Y, et al. Five year outcomes of the China National Free Antiretroviral Treatment Program. Ann Intern Med 2009;151:241-51.

Publications

List of Publication Related to Thesis Work

- S Nandi, S Maity, S C Bhunia, M K Saha. 2014. Comparative assessment of commercial ELISA kits for detection of HIV in India. BMC Res Notes 7:436. doi:10.1186/1756-0500-7-436.

  Dutta N, Nandi S, Guha S, Saha M. Emergence of HIV drug-resistant mutations in East Indian population after failure of first-line antiretroviral therapy. HIV & AIDS Review. International Journal of HIV-Related Problems. 2017;16(4):258-264. doi:10.5114/hivar.2017.72027.

  Dutta N, Omesh S, Nandi S, et al. Virologic failure on anti-retroviraltherapy without HIV drug resistance mutation. J Immune Disord Ther2018;1(1):1-5.

- Bhatta, M., Dutta, N., Nandi, S., Dutta, S. and Saha, M.K., 2020. Mother-to-child HIV transmission and its correlates in India: systematic review and meta-analysis. *BMC pregnancy and childbirth*, *20*(1), pp.1-15.

GenBank Submission

- Human immunodeficiency virus 1 msaha001459 pol gene for reverse transcriptase, complete cds
- 1,313 bp linear DNA
- Accession: LC529744.1 GI: 1820247318
- Human immunodeficiency virus 1 msaha01612 pol gene for pol protein, partial cds
- 1,300 bp linear RNA
- Accession: LC570898.1 GI: 1886868128

List of others Publication

- Banerjee S., Bhatta M., Nandi S., Dutta S and Saha M.K., 2022. Diagnostic Accuracy of HIV in-vitro Assays Evaluated by WHO Prequalification Evaluation Laboratories: Systematic Review and Meta-analysis. Japanese Journal of Infectious Diseases; JJID.2021.720

- Bhatta, M., Nandi, S., Dutta, S. and Saha, M.K., 2021. Coronavirus (SARS-CoV-2): a systematic review for potential vaccines. *Human Vaccines & Immunotherapeutics*, pp.1-18.

- Bhatta, M., Nandi, S., Dutta, N., Dutta, S. and Saha, M.K., 2020. HIV care among elderly population: systematic review and meta-analysis. *AIDS research and human retroviruses*, *36*(6), pp.475-489.

- Ghosh M Nandi S, Dutta S, Saha MK. Detection of Hepatitis B Virus infection: A systematic review. World J Hepatol. 2015 October 18; 7 (23): 2482-2491.

- Mahapatra, T., Biswas, S., Nandi, S., Ghosh, P., Ghosh, M., Mondal, S., & Saha, M. K. (2015). Burden and Correlates of HIV among Men Who Have Sex with Men in West Bengal, India: Analysis of Sentinel Surveillance Data. *PLoS ONE*, *10*(5), e0127232. doi:10.1371/journal.pone.0127232

- Chatterjee S, Nandi S, Ghosh M, Saha MK. Diagnosing the infected child: The Indian context. In: Lala MM. editor. The positive child has a right to a positive life, Action report on pediatric HIV in India. India: Abbott; 2014. P. 69 – 76.

- Maity S, Nandi S, Biswas, S Sadhukhan S, Saha M K. 2012. Performance and diagnostic usefulness of commercially available enzyme linked immunosorbent assay and rapid kits for detection of HIV, HBV and HCV in India. Virol J: 2011 9:290 doi:10.1186/1743-422X-9-290.

- Maity S, Nandi S, Biswas, S, Saha M K. 2012. Human Immunodeficiency Virus Seroprevalence among Patients Attending a Sexually Transmitted Disease Clinic in West Bengal, India. Jpn. J. Infect. Dis. 65: 424-426, 2012

Nandi et al. BMC Research Notes 2014, 7:436
http://www.biomedcentral.com/1756-0500/7/436

BMC Research Notes

SHORT REPORT Open Access

Comparative assessment of commercial ELISA kits for detection of HIV in India

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Abstract

Background: India harbors the 3rd highest HIV infected population globally. The magnitude of the HIV detection challenge is enormous. ELISA is the most commonly used screening technique for HIV. There is always an acute need for good quality ELISA kits. However, the quality evaluation data on Indian kits are very limited in comparison with internationally recognized kits. This study aimed to evaluate the performance and diagnostic usefulness of five commercially available ELISA kits which are frequently used in India.

Findings: The ELISA kits evaluated using an in-house well characterized 100 member sera panel revealed 100% sensitivity for all the batches. However, batch to batch variation in terms of specificity, positive predictive value (PPV) and efficiency, although not statistically significant ($p > 0.05$), was observed. For specificity, the 3rd generation kits (mean 99.6% to 99.3%) were comparatively better than the 4th generation assays (97.2% to 96.9%). But the 4th generation kits performed far better in the ability for early detection post HIV infection in the 25 member commercial seroconversion panel with a margin of at least 22 days and as high as 35 days than the 3rd generation assays.

Conclusions: The commercial ELISA kits with 100% sensitivity seem appropriate for HIV screening. The ability of early detection post HIV infection favors use of 4th generation kits for ensuring HIV free blood for transfusion. Lot to lot variations, especially kits having the specificity level ≤98.0%, indicate the need for a regular mechanism of kit evaluation for each batch for procuring kits appropriate for intended use.

Keywords: HIV, ELISA, Sensitivity, Specificity, Efficiency, Sera panel, Seroconversion panel

Findings

Background

HIV is a major global public health issue [1]. For assuring a safe blood supply and preventing HIV infection, proper and accurate detection of HIV is essential [2]. In India, diagnosis of HIV infection is a major challenge [3,4]. Several commercial assays are available for detection of HIV infection. ELISA is the most commonly used screening assay for HIV [2,5]. A number of ELISA kits for HIV detection with different principles are available. Nowadays, in India 3rd generation ELISA are most commonly used. The 4th generation assays are based on combined detection of antigen and antibodies simultaneously and reduce the diagnostic window period further, compared to third generation ELISA which is based on anti-HIV antibody assay [6-8]. The improved sensitivity for ELISA is mostly accompanied by a decreased specificity. In an Indian perspective, limited articles on evaluation and performance of ELISA kits are available [9] though HIV testing is being done for a vast numbers of individuals as well as large number of specimens for ensuring HIV free safe blood for transfusion. Being the 2nd most populous country with the 3rd largest burden of HIV in the world [10], the magnitude of HIV testing challenge in India is enormous and the appropriate response to the challenge is to ensure the quality of the assay kits suitable for the intended use. This study aims to evaluate the quality of commonly available commercial ELISA kits for their ability to detect HIV suitable for appropriate use in India.

Materials and methods

The study was carried out at a National HIV Reference Laboratory designated for evaluation of diagnostic kits, including ELISA, in India. A well characterized, 100 members, in-house HIV serum panel was used to evaluate and compare the performance of the kits. The sera used for

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RESEARCH PAPER

Emergence of HIV drug-resistant mutations in East Indian population after failure of first-line antiretroviral therapy

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Abstract

Introduction: There are limited data on the failure of first-line antiretroviral therapy (ART) in resource-limited settings. In particular, there have not been any reports on first-line ART failure among patients in eastern India. We review data on the emergence of human immunodeficiency virus (HIV) drug resistance among individuals with reported first-line ART failure.

Material and methods: Results of genotypic drug resistance testing were obtained from plasma samples of 44 patients who had presented with clinical or immunological failure to treatment after at least six months of ART. Major drug resistance mutations (DRMs) associated with any of the three classes of antiretroviral (ARV) drugs, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI) were seen in all patients (100% prevalence).

Results: NRTI and NNRTI DRMs were encountered at a frequency of 34 (77.27%) and 15 (34.09%) amongst 44 patients, with M184V (34.09%), T215F (25.0%), and K219E (20.45%) being the most frequent among NRTI associated mutations, and Y188L (18.18%), K103N (6.81%), and A98G (6.81%) among NNRTI associated ones. PI DRMs were observed in 5/44 (11.3%) patients, with V82L, V82S, and I84V being the commonest.

Conclusions: These results present a high prevalence of DRMs among ART patients from eastern India with clinical or immunological failure. It is very important to enhance the access of ARV drugs so that their compliance could be improved and hence development of DRMs be minimised.

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Key words: antiretroviral therapy, human immunodeficiency syndrome, DRMs, HIV-1, resistance, reverse transcriptase, protease.

Introduction

India has an estimated population of 2.11 million (1.71-2.64 million) human immunodeficiency virus (HIV)-infected individuals with an adult (15-49 years) prevalence of 0.26% in 2015 [1]. Initiated on April 1, 2004, the program of provision of free antiretroviral therapy (ART) to eligible individuals has scaled up considerably to register the second largest number of people living with HIV/AIDS (PLHA) across the globe [2]. Compared to 2000 and 2007, the Indian National AIDS Control Program (NACP) has, respectively, achieved 66% and 32% decline in new cases of HIV infection in 2015 [1]. With maturing of the program, it is essential to assess the effectiveness of ART by estimating virological

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RESEARCH ARTICLE

Virologic failure on anti-retroviral therapy without HIV drug resistance mutation

Dutta N, Omesh S, Nandi S, Saha MK *

Dutta N, Omesh S, Nandi S, et al. Virologic failure on anti-retroviral therapy without HIV drug resistance mutation. J Immune Disord Ther 2018;1(1):1-5.

BACKGROUND: HIV drug resistance (HIV DR) compromises the antiretroviral therapy (ART) outcome. This study aimed to detect presence of different HIV DR Mutations (DRM) in patients on 2nd line ART failure in Eastern India.

METHODS: HIV/AIDS patients on 2nd line ART were evaluated for virologic failure. HIV-1 genotyping was performed for the virologic failure samples employing Viroseq (Abbott Diagnostics) assay. HIVDR profile and subtype were detected using Stanford HIV sequence database following HIV DRM definition of WHO Surveillance mutation list 2009.

RESULTS: Virologic failure (HIV 1 viral load >1000 copy/ml) was detected among 15 samples out of total 365 HIV/AIDS patients (on 2nd line ART) recruited. Genotyping was successful for 9 samples having >2000 HIV/ml and remaining 6 samples could not be genotyped due to low viral copy. DR mutations were detected in 5 out of 9 samples and among the rest 4 samples no HIV DR mutation was detected. Among NRTI based drugs, M184V and M41L were the predominant mutations (80%). For NNRTI based drugs, A98G and Y181C were predominant (80%), conferring resistance to DLV and NVP. Again for PIs, l54V, A71V, V82A and M46L were seen in 40% of the cases conferring resistance to IDV, SQV, LPV, NFV and ATV.

CONCLUSION: Study on virologic failure in the absence of HIV DR mutations might help in understanding further the HIV DR dynamics as well as planning for better clinical management for patients with HIV/AIDS.

Key Words: HIV/AIDS; ART; Virologic failure; Drug resistance mutation; Reverse transcriptase inhibitor; Protease inhibitor

INTRODUCTION

Anti-retroviral therapy (ART) has successfully helped managing HIV/AIDS, but lead to emergence of HIVDR mutations [1,2]. Failing ART among people living with HIV (PLHIV) increases the possibility of emergence of HIVDR [3,4]. Failing second line ART necessitates the need of third-line ART. HIV viral load (VL) assessment is crucial requirement in ART programme. World Health Organization (WHO) advocates HIV VL monitoring to identify virologic failure as an early and precise indication of ART failure. Consequent corrective procedures would reduce accumulation of drug-resistance mutations and improve clinical outcomes [5]. However, VL testing is not routinely done in many countries due to high cost and lack of facility. HIV epidemic is nearing four decades with different ART regimens. Thus, anti-retroviral (ARV) drug resistant viruses in treatment-experienced patients are also being encountered increasingly [6]. The second line ART regimens constitute zidovudine (ZDV), lamivudine (3TC), tenofovir (TDF), and boosted lopinavir/ritonavir (LPV/r). These were introduced in India in a restricted phase wise manner [7].

The criteria to shift to second line ART are failure in clinical and/ or immunological and/ or virologic parameters in a patient on first-line ART for 6 months or more. An important target of ART is Protease (PR) that is vital for HIV replication and crucial for completion of its life cycle. Different PR inhibitors (PIs) have been approved by regulatory authorities and are in use. PIs are administered as single drug with 2 nucleoside reverse-transcriptase inhibitors (NRTIs) as second-line ART. Since 2005, boosted PIs are endorsed in combination with low-dose RTV to heighten the level of companion PIs. When PI-based ART fails, viral variants resistant to PIs emerge [8-13]. Major mutations due to amino acid substitutions alter the PR catalytic activity that consequently affects virus replication capacity [14-16]. Conversely, emergence of compensatory mutations restores replication capacity [17]. Moreover, mostly the drug resistance mutations of PR also confer cross resistance to other PIs in the class. These mutations seem essentially class specific rather than drug specific [18,19]. Boosted PI based combination ART is an efficient strategy post NNTRI-based first line ART failure. PI resistance at NNRTI-based first-line ART failure is uncommon [19].

Thus, resulting PI-based second-line ART becomes highly potent [20]. Introduction of generic first-line drugs along has considerably reduced the morbidity and mortality globally. However, in resource-limited settings, HIV viral load monitoring is rarely feasible [20]. Due to absence of VL monitoring, early virologic failure remains undetected and treatment continues to be on the failing regimen until patient develops clinical or immunologic failure resulting accumulation of drug resistance mutations [21]. High levels of HIV DR to first-line ART have been reported widely [21-24]. As a result treatment has been upgraded to PI-based second-line ART regimens in India. However, the second line ART program is somewhat limited in Indian context. Without drug resistance assay and regular virological failure monitoring, the consequences of second line therapy outcomes might remain unclear also. It is therefore, essential to assess the emergence of HIV drug resistance mutations beside clinical, virological, and immunological parameter for treatment outcomes for patients switched to second line ART. This study, first of its kind from eastern India, aimed to investigate the extent HIVDR mutations among virologic failure PLHIV on second-line ART in Kolkata.

MATERIALS AND METHODS

Study setting and population

The study was conducted at Virology Laboratory, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata. A total of 365 HIV/AIDS patients on 2nd line ART were recruited over a period of five years during January 2009 to December 2013. The study procedures followed Helsinki declaration 1975 and revision in 2000. The inclusion criteria were (i) consenting adult HIV/AIDS patients, age >18 years and on 2nd line anti-retroviral therapy and (ii) Virologic failure (plasma HIV 1 RNA level more than 1000 copies/ml) on 2 consecutive testing on 6 month interval. Exclusion criteria were if (i) blood sample was insufficient for sequencing

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BMC Pregnancy and Childbirth

RESEARCH ARTICLE Open Access

Mother-to-child HIV transmission and its correlates in India: systematic review and meta-analysis

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Abstract

Background: In India, preventing mother-to-child transmission (PMTCT) of Human Immunodeficiency Virus (HIV) remains one of the foremost challenge in community health. Countrywide MTCT of HIV is estimated to be > 10,000 annually. Aims of present study are to find out the prevalence of HIV and correlates of HIV transmission among children given birth by HIV infected mother through systematic review along with meta-analysis.

Methods: All avaiable articles are retrieved using MEDLINE, Cochrane Library, Science Direct, EMBASE, Google Scholar and PUBMED following guidelines for Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Joanna Briggs Institute Meta-Analysis of Statistics Assessment and Review Instrument (JBI-MAStARI) are applied to critically reviewing the selected articles. STATA 13.0 is used to preparation of forest plot for Meta-analysis. For assessment of heterogeneity and publication biases I^2 statistics along with Begg and Mazumdar's test and Egger's tests are used. Odds ratio (OR) along with forest plots have been showing with 95% confidence interval (CI).

Results: All together 10 studies including 1537 pairs of mothers and new births are assessed in present meta-analysis. Present analysis revealed the prevalence of HIV due to MTCT in India as 8.76% (95% CI; 5.76, 12.31). Analysis of subgroups exhibit a higher pooled prevalence in eastern region of India, 10.83% (95% CI: 5.9, 17.81) and lower in in Western region in India, 6.37% (95% CI: 4.65, 8.49). Status of MTCT before and after initiation of universal ART are 10.23% (95% CI 6.61, 14.55) and 7.93% (95% CI 4.18, 12.76) respectively. Associated factors with MTCT of HIV include absence of maternal prevention of MTCT intervention, OR = 10.82 (95% CI: 5.28, 22.17), lacking in administration of infant ARV (antiretroviral), OR = 8.21 (95% CI: 4.82, 14.0) and absence of medical facility during childbirth OR = 3.73 (95% CI: 1.67, 8.33).

Conclusions: In India, pooled HIV prevalence of MTCT as high as 8.78% (95% CI; 5.76, 12.31) among babies born to infected mothers warrants urgent need of focused intervention for providing ART (PMTCT intervention), ensuring proper infant ARV prophylaxis, and avoiding delivery without proper medical facility to pregnant women with HIV for reduction of occurrence in HIV transmission from mothers to children.

Keywords: HIV, HIV prevalence, MTCT, HIV transmission, Women with HIV, HIV-infected infant, PMTCT, India

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Original Article

Diagnostic Accuracy of Human Immunodeficiency Virus In Vitro Assays Evaluated by the World Health Organization Prequalification Evaluation Laboratories: Systematic Review and Meta-Analysis

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ABSTRACT: To maintain the performance quality, human immunodeficiency virus (HIV) in vitro diagnostic (IVD) kits are required to be evaluated by unbiased health regulatory organizations following predefined guidelines. The World Health Organization (WHO) prequalification is one such program for the evaluation of IVD assays. In the present systematic review and meta-analysis, we analyzed and compared the 17 WHO prequalified public reports of HIV IVDs to yield summarized information for performance parameters. Pooled sensitivity, pooled specificity, positive likelihood ratio, negative likelihood ratio, and diagnostic odds ratio were used as overall performance evaluation parameters. High (≥98%) and comparable levels of sensitivity and specificity were observed for most of the assays. In addition, the overall diagnostic efficiency was observed to attain high precision, as evident by the value of the area under the curve (AUC) for the hierarchical summary receiver operating characteristic curve (AUC ≥ 0.98).

INTRODUCTION

Diagnosis of human immunodeficiency virus (HIV) infection status efficiently and expeditiously is a pivotal component in global HIV management. In the emerging period of the HIV epidemic, diagnosis of HIV infection mostly relied on laboratory-based classical serological assays such as western blot and enzyme-linked immunosorbent assay (1,2). Although these methods have high diagnostic precision in confirming infection status, these costly and time-consuming assays require trained and highly skilled laboratory personnel, sophisticated equipment, and proper laboratory setup (3). In developing countries, maintaining these assays for HIV infection screening purposes is not worthwhile, especially due to the shortage of funding and the availability of well-trained laboratory staff (4). The unavailability of cost-effective and efficient diagnostic assays could lead to delayed HIV detection and treatment initiation (5). Considering this scenario, the World Health Organization (WHO) recommends the exclusive implementation of HIV in vitro diagnostic (IVDs) assays, which do not require sophisticated laboratory equipment and highly skilled laboratory personnel (6).

Various commercially available in vitro serological assays (viral antigen-antibody-based) and virological technologies (viral nucleic acid-based) are widely used for HIV diagnosis worldwide. In accordance with technological advancements, rapid diagnostic assays for HIV infection have evolved greatly and revolutionized the HIV screening procedure in many parts of the world where HIV prevalence is considerably high (7). Aside from serological assays, nucleic acid amplification test (NAAT) systems are routinely performed in many remote point-of-care centers fulfilling the purpose of HIV diagnosis (8). Although several studies confirmed the high-performing capabilities of these in vitro assays, few studies indicated some concerns regarding their efficiency compared with classical assays (9,10). In addition, lot to lot variation was reported for enzyme immunoassay-based HIV diagnostic kits (11).

According to the regional guidelines, the number of commercially available kits is increasing regularly and being used worldwide. In this scenario, regular laboratory-based evaluation of kits is necessary for quality assurance. The WHO also recommends evaluation of each new lot of IVD kits, before distribution to the diagnostic centers, by a fully functional assigned research laboratory for assuring quality (12,13). The WHO itself also initiated a

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Performance of commercially available HIV *in vitro* diagnostic assays: A systematic review and meta-analysis

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ABSTRACT

Efficient and fast detection of HIV infection is required to make the diagnosis more robust. Use of in vitro HIV diagnostic assays based on different methods are growing rapidly. To maintain quality and further upgradation, regular performance evaluation is required. Due to presence of huge number and types of commercially available kits, choice of implementation varies differentially. The present systematic review and meta-analysis is aimed to address the diagnostic performance of commercially available in vitro HIV assays in terms of pooled sensitivity, specificity, Positive Likelihood Ratio, Negative Likelihood Ratio, area under hierarchical summary receiver operating characteristic curve and diagnostic odds ratios, in global scenario. Total of 18 articles with 35 in vitro diagnostic serological assays including 29,713 samples were subjected for the present pooled analysis. In spite of higher heterogenicity [$I^2 = 93.5\%$, $Q = 520.95$, df $= 34$ ($p = 0.0000$), $\tau^2 = 9.7464$], the pooled sensitivity and specificity of the diagnostic serological assays were observed $\geq 98\%$.

1. Background

Human immunodeficiency virus (HIV) infection is an important global public health concern [1]. For conclusive detection of HIV infection, till date western blot (WB) assay is accepted as the gold standard [2, 3]. Generally, WB assay, requires more than a week to deliver a result. WB assays also require trained lab personnel for performing the assay, suitable and costly infrastructures for storage of reagents, specialized and specific equipment for conducting the test and high cost maintenance to ensure the quality of the assay [4]. But, for the developing countries, where the resource is minimal, all of these facilities are not readily available. A notable work by CDC found that approximately 25% of the study population have already reached to the fatal stage of HIV infection when they were detected. This delayed detection usually happened due to absence of reliable, accurate and fast HIV diagnostic kit [5]. Chiefly for those countries who still now rely on WB as the only confirmatory assay.

Similar argument can be made for HIV detection through ELISA. However, ELISA compared to WB is more cost efficient and less time consuming but it cannot be performed without trained lab personnel and without proper laboratory setup. Increased number of undiagnosed people unknowingly aggravate the chance of HIV infection. To combat this situation WHO recommends convenient rapid tests [6], to detect HIV antibody in whole blood/ serum/ plasma which requires no laboratory facility and no trained lab personnel [7]. Rapid HIV diagnostic kits with same day results have been introduced by WHO in different countries and regions with higher prevalence of HIV infection with lower technical and economic resources [8]. In recent years, HIV diagnosis could also be done through nucleic acid amplification test (NAAT) systems in dried blood spot (DBS) or whole blood [9]. It is based on nucleic acid extraction, amplification and identification of specific target sequences. Although NAAT can be performed in non-laboratory setups, it requires highly trained professionals to perform the test [10, 11]. Apart from these, diagnostic kits using oral fluid as assay samples instead of blood are also being popular among health care workers due to its least invasive nature and easy sample collection method. Moreover, it also completely excludes the chance of accidental needle stick injuries among clinical staff [12]. However, it is more expensive than the blood based rapid test kits and it is still a question whether its efficiency could be compared with other HIV rapid test kits [13]. Under this circumstance, WHO recommended that prior to the distribution of any diagnostic kit with new Lot/ Batch No. (despite its type or principle or manufacturer) to the testing facilities, the kit must be evaluated by an

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REVIEW

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Coronavirus (SARS-CoV-2): a systematic review for potential vaccines

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ABSTRACT

COVID-19 is an international public health emergency in need of effective and safe vaccines for SARS-CoV -2. A systematic review has been done to analyze the availability, development and status of new COVID-19 vaccine candidates as well as the status of vaccines for other diseases that might be effective against SARS-CoV-2 infection. PubMed, MEDLINE, EMBASE, Science Direct, Google Scholar, Cochrane library, ClinicalTrials.gov, Web of Science and different trial registries were searched for currently available and probable future vaccines. Articles and ongoing clinical trials are included to ascertain the availability and developmental approaches of new vaccines that could limit the present and future outbreaks. Pharmaceutical companies and institutions are at different stages of developing new vaccines, and extensive studies and clinical trials are still required.

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SARS-CoV-2; vaccine candidates; coronavirus; covid-19; clinical trial; vaccine development

Introduction

COVID-19 (Coronavirus disease 2019), a communicable illness mainly causing respiratory distress, is caused by the recently identified virus Severe acute respiration syndrome coronavirus 2 (SARS-CoV-2),[1] previously called 2019 novel coronavirus (2019-nCoV).[2] SARS-CoV-2, a single-stranded positive-sense RNA virus,[3] is mainly spread by droplets exhaled by infected persons. To date no widely available drugs or biologics for worldwide use have been shown to be effective for the prevention of COVID-19. Numerous antiviral agents, immunotherapies, and vaccines are being studied and advanced as potential therapies.[4] To date COVID-19 has caused tens of millions of confirmed cases and over one million deaths.

The Wuhan strain has been identified as a new strain of Beta-coronavirus from group 2B with ~70% genetic similarity to the previous described SARS-CoV. SARS-CoV-2 has 96% similarity to a bat coronavirus, so it is suspected to originate from bats. Vaccines had been produced for several diseases caused by other coronaviruses, including infectious avian bronchitis virus,[5] canine coronavirus[6] and feline coronavirus.[7] Previous initiatives to produce vaccines against viruses in the family Coronaviridae may provide vaccines that are clinically useful. Vaccines against severe acute respiratory syndrome (SARS)[8] and Middle East respiratory syndrome (MERS)[9] had been tested in animal models. However, no previous such coronavirus vaccine has been shown to be effective in humans.[10] According articles from 2005–6, the identity and development of new SARS vaccines become a concern for public and private health agencies around the world. At the time that MERS was predominant, it was generally believed that studies on SARS could deliver a convenient model for MERS vaccine development.[11] Only one MERS vaccine (DNA-based) finished phase I clinical trials;[12] the development of three other vaccine candidates is in progress, all as viral-vectored vaccines, two of them are adenoviral (ChAdOx1-MERS, BVRS-GamVac), and one MVA- (MVA-MERS-S) vectored.[12]

Many organizations have undertaken significant investment and research and development activities to develop a SARS-CoV-2 vaccine used the published genomic sequences.[13] The objective of this systematic review is to summarize the availability, development and status of new COVID-19 vaccine candidates as well as the status of vaccines for other diseases that might be effective against SARS-CoV-2 infection.

Coronavirus: at a glance

Coronaviruses are enveloped viruses and carry a positive-sense single-stranded RNA as genome and a nucleocapsid of helical symmetry. The genome size of coronaviruses ranges from approximately 26–32 kilobases, the largest among all RNA viruses, and they cause several diseases in mammals and birds. The symptoms in other species may vary: in chickens, they cause an upper respiratory tract disease, while in cows and pigs they cause diarrhea. Coronaviruses were first discovered in the 1930s as avian viruses during acute respiratory infection of chickens caused by an unknown infectious bronchitis virus at that time. Arthur Schalk and M.C. Hawn described the new avian virus as the possible causal agent of the respiratory infection in chickens in North Dakota in the year 1931.[14]

In humans, these viruses cause respiratory tract infections, which ranges from mild to lethal infections. Mild illnesses include some cases of the common cold (symptoms observed by the naked eye are similar to those of rhinoviruses). Some lethal varieties are SARS, MERS, and COVID-19. This group of viruses has typical club-shaped spikes (protein or glycoprotein) that project from their outer surface, which in electron micrographs create an

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HIV Care Among Elderly Population: Systematic Review and Meta-Analysis

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Abstract

Elderly people living with HIV are increasing. At present in the United States, nearly half of newly diagnosed HIV-infected people are aged >50 years. Diagnosis and treatment of HIV-infected elderly patients tends to be delayed by several health care factors as several life-threatening diseases are common in elderly people. This study aimed to find the pooled HIV prevalence in elderly population and the present situation of continuum care for the elderly HIV patients through systematic review and meta-analysis. All previously published articles from 2000 to 2018 are retrieved using MEDLINE, PUBMED, Cochrane Library, EMBASE, and Google Scholar. DerSimonian and Laird Random Effects model are used to critically appraise articles. STATA 13.0 is used to perform the meta-analysis and quantum-geographic information system (Q-GIS) is used to prepare desired map. I^2 statistics has been used to test heterogeneity and publication biases. Results have been presented using forest plots. A total of 28 studies are included in this meta-analysis. Present analysis revealed pooled prevalence of HIV in elderly population as 15.79% with a lower rate of viral suppression as 11.524% (95% confidence interval, CI: 11.199-11.855), where a moderate number 38.643% (95% CI: 38.289-38.997) of elderly patients received antiretroviral therapy (ART) globally. The ART retention rate was 12.769% (95% CI: 12.540-13.001) with 6.15% (95% CI: 6.089-6.212) mortality. Despite successful administration of ART in developing part of the world that have relatively higher retention rates among HIV-infected elderly patients only a small percentage are virally suppressed, largely due to elderly drugs interact with ART and several comorbidities reduce the life span of the elderly people.

Keywords: HIV infection; antiretroviral therapy; elderly; geriatrics; meta-analysis; systematic review.

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Detection of hepatitis B virus infection: A systematic review

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Conflict-of-interest statement: All the authors declare that they have no competing interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at sahamk@yahoo.com. No additional data are available.

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Abstract

AIM: To review published methods for detection of hepatitis B virus (HBV) infection.

METHODS: A thorough search on Medline database was conducted to find original articles describing different methods or techniques of detection of HBV, which are published in English in last 10 years. Articles outlining methods of detection of mutants or drug resistance were excluded. Full texts and abstracts (if full text not available) were reviewed thoroughly. Manual search of references of retrieved articles were also done. We extracted data on different samples and techniques of detection of HBV, their sensitivity (Sn), specificity (Sp) and applicability.

RESULTS: A total of 72 studies were reviewed. HBV was detected from dried blood/plasma spots, hepatocytes, ovarian tissue, cerumen, saliva, parotid tissue, renal tissue, oocytes and embryos, cholangiocarcinoma tissue, *etc*. Sensitivity of dried blood spot for detecting HBV was > 90% in all the studies. In case of sero-negative patients, HBV DNA or serological markers have been detected from hepatocytes or renal tissue in many instances. Enzyme linked immunosorbent assay and Chemiluminescent immunoassay (CLIA) are most commonly used serological tests for detection. CLIA systems are also used for quantitation. Molecular techniques are used qualitatively as well as for quantitative detection. Among the molecular techniques version 2.0 of the CobasAmpliprep/CobasTaqMan assay and Abbott's real time polymerase chain reaction kit were found to be most sensitive with a lower detection limit of only 6.25 IU/mL and 1.48 IU/mL respectively.

CONCLUSION: Serological and molecular assays are predominant and reliable methods for HBV detection. Automated systems are highly sensitive and quantify HBV DNA and serological markers for monitoring.

Key words: Chemiluminescent immunoassay; Serology; Automated detection; Molecular assay; Hepatitis B virus

Core tip: The article was aimed to review published

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RESEARCH ARTICLE

Burden and Correlates of HIV among Men Who Have Sex with Men in West Bengal, India: Analysis of Sentinel Surveillance Data

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Abstract

Background

Little is known about the socio-behavioral risk factors for HIV acquisition among hard-to-reach men who have sex with men (MSM) population in India, particularly from the densely populated eastern part. Thus to measure the burden and correlates of HIV among MSM in West Bengal state of eastern India, a cross-sectional analysis of the national HIV Sentinel Surveillance (HSS) data was conducted.

Methods

In 2011, between July and September, involving all sentinel sites of the state, 1237 consenting MSM were anonymously interviewed and tested for HIV following national guidelines. Using a short, structured questionnaire, information was collected on socio-behavioral factors along with sexual practices and was analyzed to determine burden of HIV and the role of its socio-behavioral correlates on HIV acquisition.

Results

Among participants, mean age was 23.4 years, 44.55% were "Kothis" (usually receptive partner) and 25.1% admitted receiving money for sex with man. HIV sero-positivity was 5.09%. Using logistic regression method, for both bivariate and multivariate (with saturated model) analyses, transport-workers [adjusted odds ratio (AOR)=8.95, 95% confidence interval (95%CI): 1.09-73.71), large business-owners/self-employed (AOR=8.46, 95%CI: 1.25-57.49), small business-owners/cultivators (AOR=7.90, 95%CI: 1.67-37.38), those who visited the sentinel site for official purposes (AOR=7.60, 95%CI: 1.21-47.83) and paying money for having sex with men (AOR=3.03, 95%CI: 1.10-8.33) were strongly associated with higher HIV sero-positivity with than their counterparts. Using the parsimonious model for multivariate analysis, Kothis (AOR=4.64, 95%CI: 1.03-20.89), paying (AOR=2.96, 95%CI:

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Performance and diagnostic usefulness of commercially available enzyme linked immunosorbent assay and rapid kits for detection of HIV, HBV and HCV in India

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Short Communication

Human Immunodeficiency Virus Seroprevalence among Patients Attending a Sexually Transmitted Disease Clinic in West Bengal, India

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SUMMARY: Sexually transmitted infections (STIs) such as human immunodeficiency virus (HIV) infection have become a major public health problem globally as well as in India. Prevalence of STIs varies across different high risk groups including the population with sexually transmitted diseases (STDs). Individuals with STDs are at a higher risk of acquisition of HIV through the sexual route than the other routes. The tendency of HIV infection was studied in attendees aged 15–49 years in an STD clinic in West Bengal from 2004 to 2008. Blood samples were collected consecutively from 250 attendees per year (a total of 1,250 samples over 5 years) from an STD clinic during 12 weeks (October-December) every year. HIV sero-status was screened using ELISA, and positive samples were subjected to rapid assay and confirmed by Western blot. Overall HIV seropositivity in STD patients was 1.28% (women, 1.04%; men, 1.48%). Data from 5 consecutive years showed a prominent decline from 2.40% (2004) to 0.0% (2007) and a minor increase (1.6%) in 2008, which was not statistically significant ($\chi^2 = 4.6$, df = 4, $P > 0.05$). The highest seroprevalence (1.66%) was observed in the 25–29 age group, and the lowest (0.87%) was observed in the 40–44 age group. The overall decreasing tendency of HIV infections signifies the efficiency of current interventions. Focused intervention for the 25–29 age group may help in decreasing HIV infections further.

Human immunodeficiency virus (HIV) is one of the most important sexually transmitted pathogens and a major public health concern for sexually transmitted infections (STIs) globally (1–5). An estimated 34 million (31.6–35.2 million) people worldwide were living with HIV infection and/or AIDS at the end of 2010 (6). Globally, approximately 2.7 million (2.4–2.9 million) people were newly infected in 2010 (6) as compared to approximately 2.6 million (2.3–2.8 million) new infections in 2009 and 3.1 million (2.9–3.4 million) in 1997; this indicates a declining trend of new HIV infections (7). Moreover, a decreasing trend in individuals with HIV and/or AIDS as well as a decline in new HIV infections was observed in 33 countries (7). However, an earlier report reveals a significant increase in the rate of sexually transmitted diseases (STDs) as well as HIV infections in STD populations (8,9); further, more individuals among the urban population were affected by HIV than those among the rural population (1,8). Additionally, HIV seroprevalence varied with the marital status of the attendees (1,8). The HIV infection rate in 2009 varied significantly in different countries globally as follows: 5.0%, sub-Saharan Africa; 0.2%, North Africa and the Middle East; 0.3%, South and South-East Asia; 0.5%, North and South America; 0.8%, Eastern Europe and Central Asia; and 0.2%, Western and Central Europe (7). In India, HIV infections emerged in the mid 1980s, and there was a rapid increase in its incidence and prevalence (10). Unprotected sex is the major route of HIV transmission, and it contributes to approximately 88.7% (87.4% heterosexual and 1.3% homosexual) of the estimated HIV infections in India (11).

In 2009, the National AIDS Control Organization (NACO) estimated that 2.39 million (1.93–3.04 million) people were living with HIV infections and/or AIDS in India, with the estimated new infections and adult prevalence being 0.12 million and 0.31%, respectively (11,12). In India, among patients attending an STD clinic, seropositivity of HIV was estimated to be 1.4% –14% with a higher incidence observed among commercial sex workers (CSWs) than the non-CSWs in 1995 (13). It has been reported that there is a high prevalence of HIV transmission among high-risk populations of CSWs, patients at STD clinics, intravenous drug users, and commercial blood donors in India (14); moreover, a high incidence of HIV infections was observed in STD clinic attendees (15,16). Earlier reports have estimated that HIV seropositivity among STD clinic attendees in Calcutta, West Bengal (17–19) during 1988–2000 was approximately 1.3%–2.3%; however, there are no reports on the current scenario.

The present study aims to understand the occurrence of HIV in both male and female patients of different age groups attending an STD clinic of West Bengal.

Blood samples from 250 attendees were collected within a time span of 12 weeks (October-December) every year from 2004 to 2008 (total, 1,250 samples) in a consecutive manner, and an unlinked anonymous testing strategy was followed. An aliquot of unused serum was taken and given only a code number such that there

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