# Elucidation of Host and Viral factors in HIV-1 infected individuals.


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# Elucidation of Host and Viral factors in HIV-1 infected individuals.

A Thesis submitted in partial fulfillment of requirement for the degree of

Master of Sciences

In

Healthcare Biotechnology

By

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**2017**

# DECLARATION

I hereby declare that my Master's thesis entitled "Elucidation of Host & Viral factors in HIV-1 infected individuals" is solely my own work and has been written independently with no other aids and sources than those quoted.

_____

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December, 2017

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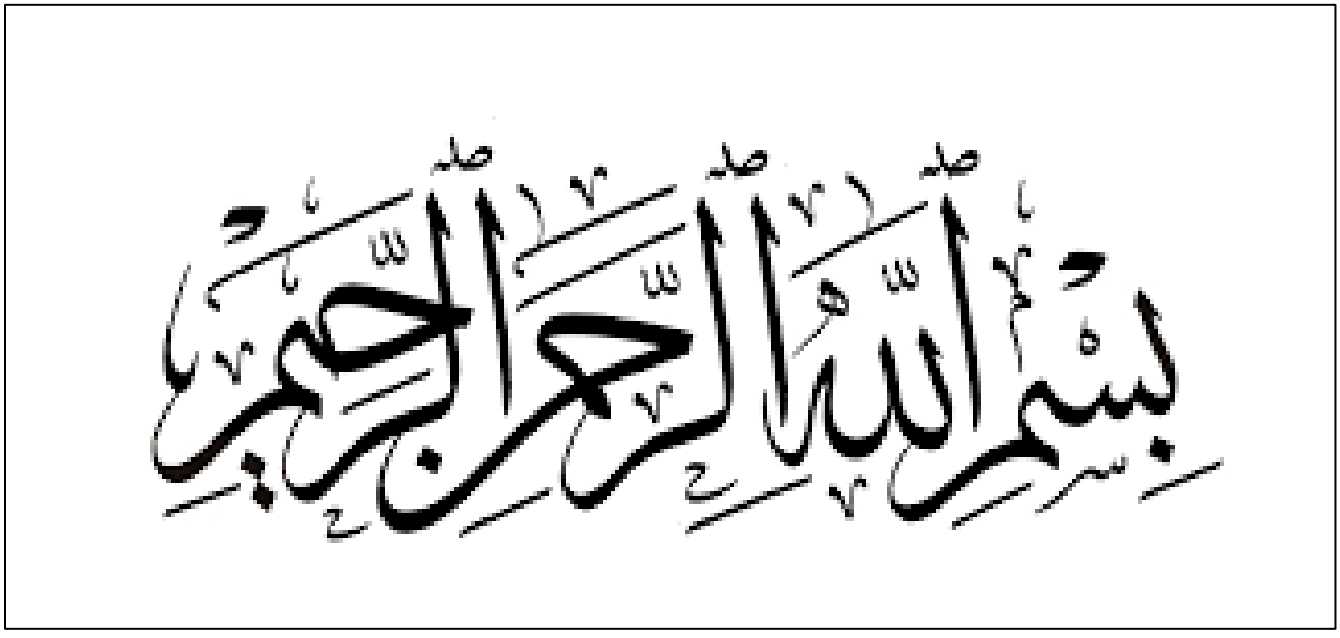
بسم الله الرحمن الرحيم

*“We will show them Our signs in the horizons and within themselves until it becomes clear to them that it is the truth. But is it not sufficient concerning your Lord that He is, over all things, a Witness?”*

(Quran 41:53)

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# *LIST OF ACRONYMS*

| | |
|---|---|
| % | Percentage |
| > | Greater than |
| < | Less than |
| AIDS | Acquired Immunodeficiency Syndrome |
| ART | Anti-retroviral therapy |
| bp | Base pair |
| CCL | Chemokine (C-C motif) ligand 3 |
| CCR5 | C-C chemokine receptor type 5 |
| CD | Cluster of Differentiation |
| CDC | Center for Disease Control |
| CXCR4 | C-X-C chemokine receptor type 4 |
| DB | Database |
| dNTPs | Deoxynucleotide Triphosphates |
| EDTA | Ethylene-diamine-tetra-acetic Acid |
| FDA | Food and Drug Administration |
| gp | Glycoprotein |
| GWAS | Genome-wide Association studies |
| HapMap | Haplotype Map |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigen |
| IDUs | Injection Drug Users |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |

| | |
|---|---|
| Kb | Kilobases |
| LD | Linkage Disequilibrium |
| LTNPs | Long-term Non-progressors |
| $MgCl_2$ | Magnesium Chloride |
| mL | milli-Liter |
| mM | milli-Molar |
| $mm^3$ | Cubic milli-meter |
| NACP | National AIDS Control Program |
| NCBI | National Center for Biotechnology Information |
| PCR | Polymerase Chain Reaction |
| RT | Reverse Transcriptase |
| SDS | Sodium dodecyl sulfate |
| SIV | Simian Immunodeficiency Virus |
| SNP | Single Nucleotide Polymorphism |
| ss | Single Stranded |
| SSP | Sequence Specific Primers |
| TAE | Tris-Acetate-EDTA |
| TE | Tris-EDTA |
| TGF-β | Transforming growth factor beta |
| TRIM-5α | Tripartite motif-containing protein 5 |
| µL | Microliter |

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# *Abstract*

In the last three decades, the world has known HIV/AIDS as a disease that has been responsible for one of the major anthropological epidemics our globe has faced since the time it was discovered. From the time HIV/AIDS was initially made known in 1982 and HIV revealed in 1983, marvelous advancement has been achieved in knowing the source of its evolutionary tree as well as knowing the fundamental biology of the this virus, the intricacies of its communication with the complex immune system of humans, and the various ways accountable for its spread, early propagation, and pathogenesis.. The role of host factors such as genetic single nucleotide polymorphisms' (SNPs) and the viral sequences in the variance seen in susceptibility to HIV infection, viral spread, immune response to therapeutic interventions and the pace of progression to AIDS is established in other populations. However, no such study is available from Pakistani population. For this purpose, three cytokines IL-10, TGF-β and IL-18 were chosen for investigating their association with HIV pathogenesis in Pakistani patients. The viral integrase and part of RNAse H gene was also sequenced. A possible association was sought for 40 HIV-1 patients and controls for IL-18 -607C/A, IL-10-1082A/G, and TGF-509C/T polymorphisms. The SNPs were verified using allele specific PCR as well as restriction fragment length polymorphism analysis (RFLP). A 161 and 700 amplicon within the integrase gene was also optimized using in-house PCR and thereafter sequenced. The study found that there was no significant difference amongst the HIV patients and control for polymorphisms in IL-10 and IL-18, however higher IL-10-GG genotypes were found in the control group which

is linked to faster progression to AIDS as well as higher IL-18-CC/CA genotypes amongst both the groups which is also linked to rapid progression to AIDS. Moreover, a higher TGF-β -509TT genotype and T allele was found in half of the HIV patients suggesting its strong association with a high viral load. The 161bp and 700bp amplicons were successfully amplified from all HIV positive patients for designing of a future in-house HIV-1 detection assay as well as few 700bp amplicons were sequenced for future phylogenetic analysis. These results may aid in future cohort studies as well as genotyping, diagnostics and drug resistance mutation analysis.

# *Chapter 1*

# *Introduction*

The worldwide HIV/AIDS epidemic continues to exact an massive toll, taking 1.3 million lives in 2014 and 35 million from the time when AIDS was documented almost 30 years ago. According to recent statistics, globally 38 million people live with HIV/AIDS with 1.8 million novel infections and 1.1 million expiries in 2016 (WHO, 2017). In Pakistan alone, there are roughly 0.133 million people breathing with HIV infection (NACP, 2016). In the face of these unnerving figures, the worldwide disposition of established treatment and deterrence approaches has slackened the assault of HIV/AIDS, which has reduced the number of deaths and incident infections by almost 33% in the last 10 years. These accomplishments did not come without difficulty. They were the outcome of many years of novelty commencing with essential rudimentary studies that resulted in fruitful interferences. Undeniably, rudimentary investigation on HIV/AIDS is inevitably related to the progress of operative interferences for the ailment (Tebas, et al., 2014).

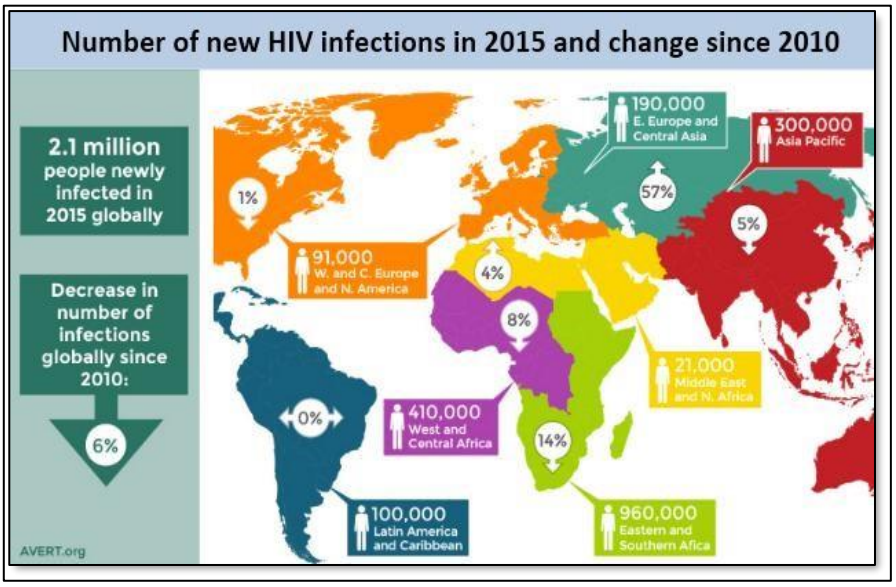



***Figure 1.1***. *Geographical distribution of new HIV infections worldwide.*

*Source: (UNAIDS, 2017)*

HIV has arisen as the utmost pandemic in the contemporary history of health sciences. HIV is a lentivirus that binds to the Helper CD4+ T lymphocytes via its glycoprotein receptors, and by this means initiates a straight bout on the adaptive immune system. Additionally, being a retrovirus, this virus perseveres for the reason that reverse transcription fallouts in the incorporation of DNA genomic duplicates which continue to remain dormant in latent CD4+ T cells. Furthermore, vigorously duplicating virus particles can persevere thanks to its capability to produce genetic mutants that can evade antibodies designed against the virus or active T-lymphocytes. Obstinate infection is escorted by chronic unnecessary immune stimulation, due to the movement of microbes present in the intestine. Sooner or later, the outcome is immune collapse, elucidating the inconsistent reduction in the levels of non-infected cytotoxic CD8+ T lymphocytes (Lane HC, 1985). Moreover, Immune compromise results in opportunistic infections along with neoplasms, which are unvaryingly deadly if antiviral therapy is not initiated (Al-Jabri, 2007). It has been shown through studies that when African monkeys were infected with their natural SIV virus, the high viral load did not account for the pathophysiology of HIV - in other words, it is not only the HIV/SIV but host response to these viruses as well that elucidates AIDS in either simians or human species. It looks as if it has been spread to humans in a minimum of 4 discrete occurrences, documented by separate HIV-1 ancestries named groups (the M, N, O, P) (Kaur & Nehra, 2009). Of these, the most substantial group of HIV-1 is the M group, a group has been accountable for the immense widely held human infections. HIV-1 M group can then be further can be divided into at least eleven subtypes classified A1-A4, B, C, D, E, F1/F2, G, H, J and K11 subtypes; O group has O subtype, N group has N subtype. HIV-2 has at least A, B, C, D, E, F and G7 subtypes. The HIV subtypes are most

important in etiology, epidemiology, laboratory diagnosis, clinical symptoms, drug screening and evaluation as well as vaccine development.

In fact, there is a marked difference in the probability of transmission of sexual contact between HIV subtypes. Studies have shown that there are indeed differences in the transmission of different HIV-1 subtypes under specific conditions. In recent years, the epidemic of HIV-1 subtypes has shown a new trend. The subtype A is predominantly found in the south of the sub-Saharan Africa and is now being replaced by subtype C; Subtype B is predominant in Thailand's intravenous drug users, but the proportion of newly infected A-type infections is rapidly rising; In China and India, most infections which were previously identified as HIV-1B subtypes and are now subtype C through recombination's. Changes in the proportions of HIV-1 subtypes suggest that the transmission of HIV-1 subtypes may vary (Erickson & Burt, 1996).

Various experiments on HIV infection and pathogenesis support all HIV investigations, where the field of therapeutics on top of the list. Comprehensive information of the duplication cycle of HIV opned the doors for new exciting targets for anti-HIV drugs. Researches have produced greater than thirty approved Antiretrovirals and Antiretroviral blends, wholly reliant on an close information of the viral reproduction cycle (Clavel & Hance, 2004). By June 2017, 20.9 million people were getting Antivirals, preventing around 8 million projected deaths from 2001 to 2017 (data courtesy of UN Joint Programme on HIV/AIDS (UNAIDS), 2017). Antivrials have likewise transformed HIV deterrence, particularly via stoppage of mother-to-child spread (PMTCT) plans, thereby avoiding 1.5 million cases sine 1999 (UN Joint Programme on HIV/ AIDS (UNAIDS), 2016).

Our genotype interacts with the external environment to generate changes in disease vulnerability and pathogenesis at individual level. In recent years, the Genome-Wide Association Studies (GWAS) have led to the identification of single nucleotide polymorphisms (SNPs) linked to a variety of diseases that have earlier not been assessed for their disease significance. SNPs are used in mapping diseases to various genes, as genetic biomarkers and pharmacogenomic targets for drug treatments (Fareed & Afzal, 2013). They occur both in the coding as well as non-coding regions of the genome and influence an array of loci located close to them.

The GWAS catalog, which is a curated assemblage of all published genome-wide association studies, has more than a hundred-thousand SNPs reported. It is curated manually, and its quality is strictly monitored. More than 200 diseases have been studied for GWAS and above 1200 studies are reported. HIV SNPs have been shown to be linked to the difference in disease susceptibility, viral load in the plasma and reservoirs, success of treatments regimen, HIV pathogenicity, and other related factors. Several studies have been conducted for assessment of polymorphisms in HIV associated genome regions, but to date, no published literature is available for such studies evaluating the globally reported SNPs within the coding and non-coding regions (Welter, et al., 2013).

Consequently, this study was undertaken to identify the SNPs prevailing within the Pakistani population as well as to identify the strains of HIV-1 circulating within the Pakistani population.

The main objectives of this study include:

1. To genotype SNPS reported in literature having association with HIV/AIDS within the Pakistani Population

2. Establish a simple PCR assay for the detection and genotyping of HIV

# *Chapter 2*

# *Literature Review*

# 2.1 Human Immunodeficiency Virus Infection

In the past thirty years, HIV/AIDS has instigated one of the main human crises our earth has ever witnessed to date. HIV/AIDS has been described as the foremost "mighty plague" of the 20th and 21st centuries. As a multifaceted and unfathomable instance of viral induced pathogenesis, SIV along with HIV have stood the topic of extra exhaustive investigation as compared to other human or animal pathogenic virus. As AIDS was foremost pronounced in 1981 and the causative agent virus or HIV revealed in 1983, incredible advancement has been done in undermining the ancestral lineage and origin of this virus as well the source and elementary virology, the intricacies of its communication with the immune structure of the host, in addition the processes accountable for its spread, primary propagation, and virulence.

## 2.1.1 Epidemiology

Even though HIV was perhaps foremost conveyed from apes to humans in Africa in the early 1930s, it first presumed epidemic status around the 1970s in Africa and nearly 10-20 years later far along in other areas of the Globe. It is appraised that AIDS has triggered nearly 40 million demises starting the time when this epidemic commenced, and that greater than 36.8 million people apart from this are at present breathing with this disease, whereas there are greater than 2 million novel infections annually (UNAIDS, 2017). According to UNAIDS data collected in 2017, 20.9 million people are alive on antiretroviral therapy in more than 160 countries around the world. In 2016 alone, there were 1.8 million new HIV infections, making the overall number of people living with HIV around 36.7 million (UN Joint Programme on HIV/ AIDS (UNAIDS), 2017). According to the data collected in 2016 from Asia and Pacific, 5.1 million people were

alive with HIV. 270,000 new HIV infections were contracted, 2.4 Million people with HIV were on antiretroviral treatment and 170000 AIDS-related deaths occurred (UNAIDS; GARPR, 2016). In some of the nations with the uppermost occurrence of HIV contaminations, it is anticipated that AIDS caused a alarming reduction in the overall life expectancy, an effect that may well be exclusive amongst viral sicknesses. Life expectation in Botswana was abridged from a non-AIDS estimate of 67 years to 44 years, preceding to a huge availability of antiviral treatment (Jing, et al., 2014). One more infrequent aspect of the AIDS epidemic is its accumalation in juvenile adults who are the main locomotives of society. This stage of development choice has fashioned a huge set of orphans and destroyed the earnings and bondness of kin, the societal element that is a main column of humanity. Young females between the ages of 16 and 25 are at an increased danger of infection accounting for 25% of the new global HIV infections in 2016 in spite of the fact that they constitute only 10% of the total population. Moreover, considering the worldwide HIV statistics, 79% of the HIV positive pregnant females were granted access to treatment for deterrence of child infection during labor (World Health Organization, 2017). Figure 2.1 shows the status of people living with HIV across the globe.

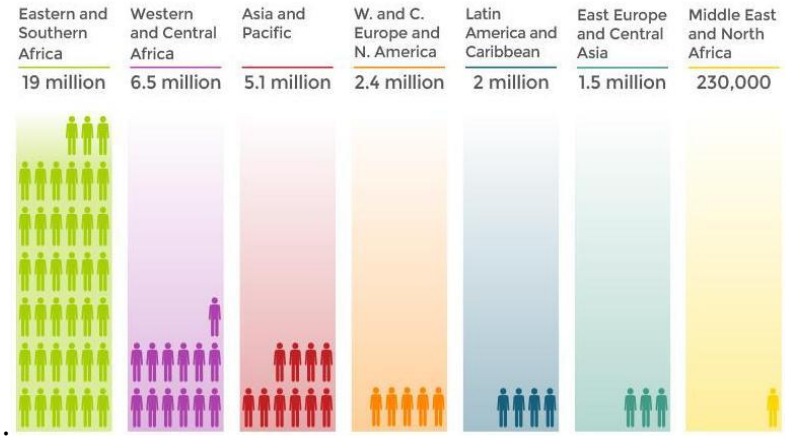
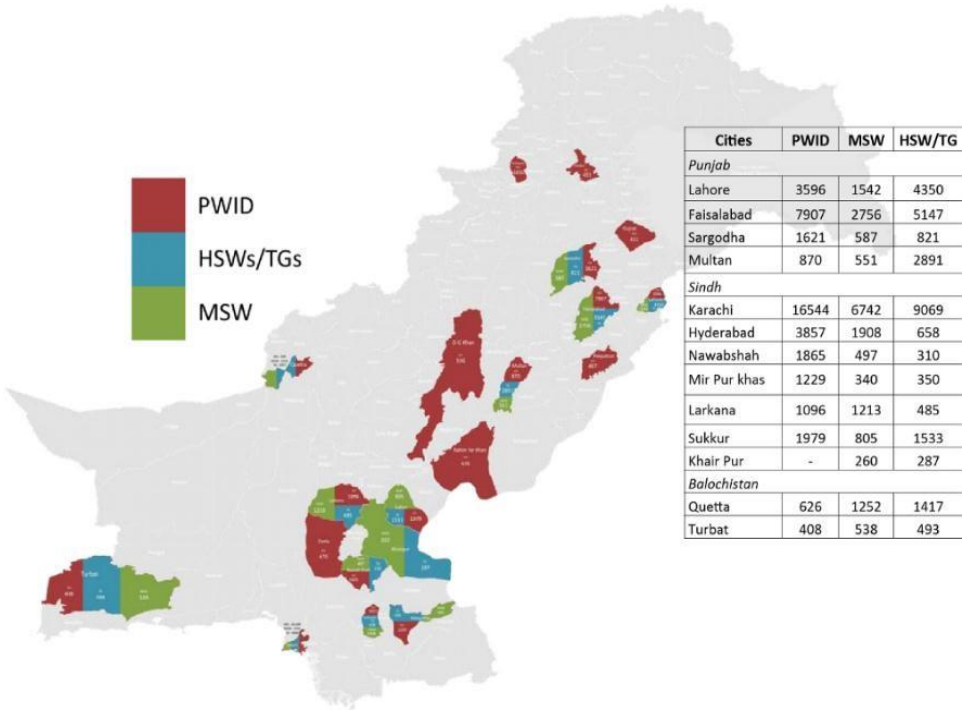



***Figure 2.1***. *Number of people living with HIV in 2015. Source: UNAIDS (2017) URL: https://www.avert.org/infographics/number-people-living-hiv-2015*

## 2.1.2 HIV/AIDS in Pakistan

In Pakistan, Pakistan is listed amongst the few countries in the entire Asia where the incidence of AIDS is increasing since the 1990s. As of 2016, there are 130,000 cases of HIV in Pakistan and only 21,575 are registered with the 21 government-run HIV-treatment centers in Pakistan NACP, 2016). Of these, 11,541 are on antiretroviral therapy (ARTs). Each year there are 19,000 new cases and 5500 deaths (NACP, 2016). The incidence is not directly measured. Instead spectrum modeling and AEM modeling is used. There are four major population HIV-risk groups in Pakistan. These include People Who Inject Drugs (PWID) with a prevalence of 38.4%, followed by Hijra Sex Workers (HSWs) at 7%. Men-who have-Sex with Men (MSMs) form 5.4% and Female Sex Workers (FSWs) form 2.2% (Country Coordinating Mechanism, 2016).




| Cities | PWID | MSW | HSW/TG |
|---|---|---|---|
| *Punjab* | | | |
| Lahore | 3596 | 1542 | 4350 |
| Faisalabad | 7907 | 2756 | 5147 |
| Sargodha | 1621 | 587 | 821 |
| Multan | 870 | 551 | 2891 |
| *Sindh* | | | |
| Karachi | 16544 | 6742 | 9069 |
| Hyderabad | 3857 | 1908 | 658 |
| Nawabshah | 1865 | 497 | 310 |
| Mir Pur khas | 1229 | 340 | 350 |
| Larkana | 1096 | 1213 | 485 |
| Sukkur | 1979 | 805 | 1533 |
| Khair Pur | - | 260 | 287 |
| *Balochistan* | | | |
| Quetta | 626 | 1252 | 1417 |
| Turbat | 408 | 538 | 493 |

***Figure 2.2****. HIV risk population distribution in Pakistan. Adopted from "Country Case Study Report for UNAIDS, 2013"*

In distinction to numerous other viral pandemics, HIV does not diminish by wearing vulnerable hosts, instead it is spread by the repeated enrolment of juvenile grownups as they develop in to sexually vigorous beings. Additionally, the virus is spread by numerous means- via blood and blood related products, by usage of syrignes and needles that have been contaminated or re-used, from moms to their neonatal, apart from sexual interaction. Lastly, HIV new infections tend to to aggregate in societal subclasses that can be disparaged, such as professional paid sex labors, injecting drug users or IDUs, and homosexuals. Altogether, these features have transformed the plague in to a intimidating communal well-being trial (Williams, et al., 2006).

### 2.1.3 Viral Classification

SIV and HIV both belong to the lentivirus affiliates of the *Retroviridae* family of viruses. SIVs are the endogenous lentiviruses of primate species, and utmost—maybe all—species of African simians have their "particular" strain of SIV virus. These SIVs are communicated horizontally, probably by sexual copulation, grooming, aggressive, or other contacts of close proximity (Sharp & Hahn, 2010). Even though they are the reason for lifetime insistent infections in their usual hosts, along with high virus titers, they do not seem to cause any disease on its own (Clements & Zink, 1996). There are 2 main clusters of human lentiviruses, HIV-1 and HIV-2, together which instigate from spread of Simian immunodeficiency viruses from nonhuman primates. HIV-1 was a consequence of  a transfer of chimpanzee virus (SIVcpz), while HIV-2 was attained by means of infection of a sooty mangabey virus (SIVsmm) (Månsson, et al., 2014).  HIV-1 strains are the major cause global epidemics and most of the world's AIDS patients are infected with HIV-1 strains; those infected with HIV-2 strains are concentrated in only a few West African countries with less clinical signs than HIV -1 infection and

comparatively have a longer average life expectancy. HIV-1 was further divided into three groups according to the homology of their gene sequences: major (M), Outlier (O), and N (new or non-M, non-O), each originating from a dissimilar cross-species transmission incident (Lemey, et al., 2013).

HIV-1 strains that infect the vast majority of infected persons in the world belong to group M, and only a small group of infected persons in central Africa are infected with group N and group O (Araújo & Almeida, 2013). M group HIV-1 contains 9 subtypes (clades) including A, B, C, D, F, G, H, J, and K. In addition, the circulating recombinant form (CRF) virus is an intra-subtype or sub-type recombinant formed during an infection with multiple HIV-1 strains leading to the recombination of the virus genome (Jacobs, et al., 2014). Currently, there are CRF reports all over the world and 80 CRFs have been confirmed. CRF01-AE is the earliest identified recombinant subtype that was originally isolated in Thailand and is a major pandemic strain in Southeast Asia. Sub-Saharan Africa is the fastest-growing HIV region, with the most diverse forms of epidemics in the region. The epidemic recombinant CRF18 cpx identified in Cuba was associated with 38 different HIV strains, most of which came from Africa. Epidemic recombinant type of viruses formed due to recombinant gene mutations in the virus, change the biological characteristics of the virus, including the immune response and anti-viral drug sensitivity. Due to virus variation and recombination, HIV-1 viruses of different subtypes are distributed throughout the world (F.Santos & Soares, 2010). C subtype HIV-1 infections account for 50% of the total HIV-infected persons in the world and are the most prevalent strains in the world; The subtypes B are mainly distributed in the United States, Europe and Australia. Subtypes A are mainly distributed in eastern Africa, eastern Europe and central Asia. Subtype C is mainly distributed in southern and eastern Africa as well as in India and China, Nepal and

other population more densely populated areas (Hemelaar, et al., 2011). Africa is the region with the most HIV subtype distribution and almost all HIV subtypes. Epidemic strains in different regions of each continent are not the same. For example, the epidemic strain in Central Asia is A subtype, while in East Asia is C subtype. Southeast Asia is CRF01-AE recombinant subtype. On the other hand, the patterns of geographical distribution of HIV-1 subtypes are constantly changing with the modes of transmission and immigration. In eastern Europe, the virus of subtype B was once the predominant strain, but a 2003 survey found that the A1 subtype virus has become the predominant strain in the area. In Pakistan too, subtype C is more prevalent (Zhang, et al., 2010). The major epidemic strains in many parts of the world are constantly evolving with social, economic, cultural and health factors, which are also one of the key features of the HIV epidemic.

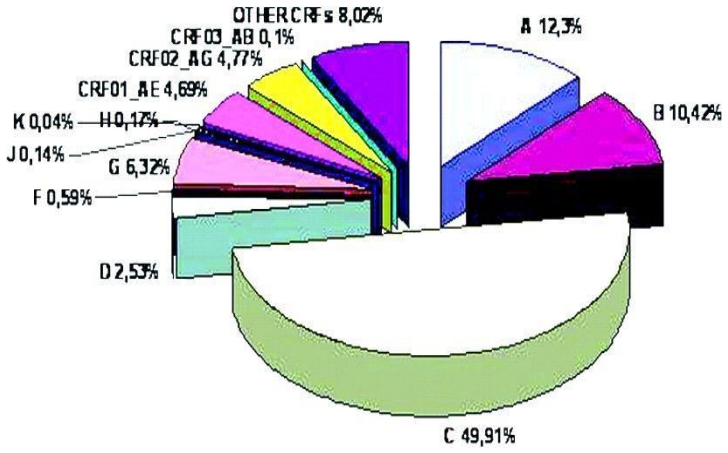



***Figure 2.3**. Global prevalence of HIV-1 genetic forms. Adopted from (Buonaguro, et al., 2007)*

## 2.1.4 Viral Structure and Genome

The genome of HIV is a single-stranded, positive sense RNA of 9.7kb (Harvey, 2007). It is composed of 3 chief genetic loci in mutual amongst all retroviruses: gag translates into the core

proteins that cover the RNA genome, while pol codes for the enzymes Protease, reverse transcriptase (RT), integrase, and env codes for the envelope glycoprotein (POZ, 2016). Figure 2.4 displays the HIV-1 genome organization.

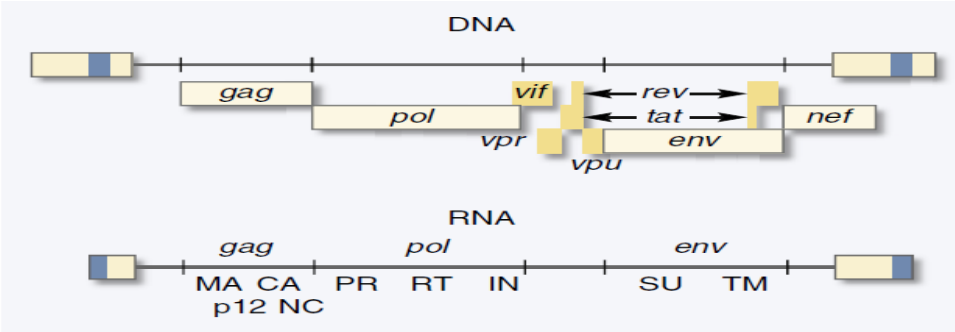



***Figure 2.4.*** *HIV-1 genome organization. The RNA genome is around 9.2 kb in length and is circumscribed at the two corners by a noncoding repeat (R) region that encompasses the 3 main coding genes, the gag (group antigen), pol (polymerase), and env (envelope) genes resulting in dissimilar reading frames as a result of transcription. The illustration specifies the location of the main proteins coded by every one of these gene. These include the MA (matrix) followed by p12, then CA (capsid), after which comes NC (nucleocapsid) proteins of gag, then the PR (protease), next is RT (reverse transcriptase) followed by IN (integrase) enzymes of pol, and finally the SU (surface) and TM (transmembrane) proteins of env. HIV-1 also encodes six nonstructural accessory proteins (tat, rev, vif, vpr, vpu, and nef) whose open reading frames are illustrated.*

## 2.1.5 Subtyping

Subtyping restructuring is an important way for HIV to evolve. At the same time, the recombinants have more complex characteristics and transmission efficiency than the single type strains, and gradually show the tendency to replace the single subtype. Knowing the genetic subtypes of Human Immunodeficiency Virus type I (HIV-1) has been very beneficial for tracing the origin and worldwide spread of this virus. Subtyping may also aid in determining the pathogenicity of the virus and as well as shed light on the possible routes of transmission and future targets for drug and vaccine designing (Araújo & Almeida, 2013). The main method for subtyping involves sequencing the full-length genome of the virus or either a specific region of

the genome such as envelope or the polymerase gene that is heterogenous enough to distinguish between the subtypes yet conserved. Most of the genetic variability is within the env gene, which codes for the envelope (Env) surface glycoprotein 120 (gp120) and transmembrane glycoprotein 41 (gp41), with 35% inter-subtype and 20% intra-subtypes variation (Girard, et al., 2011).

## 2.2 GENETIC DETERMINANTS OF HOST SUSCEPTIBILITY TO HIV

Individuals that are infected with HIV can be grouped into numerous groupings depending on their CD4+ T lymphocyte levels, the level of viremia, and stretch to final AIDS (Girard, et al., 2011). A subgroup known as Rapid progressors usually have greater viral set points, fast exhaustion of outlying CD4+ T cells, and progress to AIDS in less than years from the preliminary infection. Another infrequent subset (<3%) of HIV infected patients so called long-term nonprogressors, slow progressors, or HIV controllers (HIC), continue to be symptom fee for a minimum period of at least ten years, but habitually considerably lengthier, in the absenteeism of Antiretrovirals, with steady CD4 cell amounts (average 450-500 cells/uL), and highly reduced levels of plasma viremia. Apart from this, even a scarcer subgroup (<0.5%), are persons who have untraceable viral load in the absenteeism of antiretrovirals. These HIV infected individuals are every so often termed as elite controllers (ECs). Even furthermore, An additional tremendously infrequent subgroup of HIV-infected people are termed as viremic nonprogressors (VNPs), that is in which steady CD4+ T lymphocyte counts are upheld regardless of comparatively greater quantity of viremia. These VNPs display aspects analogous to those noticed during infection of normal hosts by non-infectious SIVs an example being the sooty mangabeys (SMs), together with the nonappearance of chronic immune stimulation and

reduced quantity of diseased central-memory CD4+ T lymphocytes (Carrington, et al., 2001). The capability of the host to hold an HIV infection indeterminately is vital to knowing the changing aspects of contagion and is frequently dependent on the specific strain of the virus, environmental factors and the genetic variants within the array of host genes. There are many genetic determining factors of the host that effect vulnerability to infection or the speed with which a person progresses to AIDS. These factors mostly can be divided into 2 groups, those that modify co-receptor accessibility and those in HLA loci. (Liu, et al., 1996). Most of these host genes are responsible for producing an array of cytokines both proinflammatory (TNFα, IL-1, IL-6, IFNγ, and IL-8) and anti-inflammatory (IL-13, IL-10, IL-14, IL-1ra, and TGFβ) some of which govern the quantitative level of co-receptors or co-receptor ligands expression (for example chemokines) that can lessen receptor accessibility for attachment of HIV virus such as (RANTES, CCR5, SDF1, and CCR2) while others are involved in immune modulation (TNFα, IL-10, MBL) (Kaur, et al., 2013). The assortment of HIV peptides for immune presentation is governed by HIV alleles (Goulder, 1997). Particular HLA alleles (i.e., HLA B27 and B57) are linked with deferred advancement to AIDS and improved virus curbing, while others are linked with added fast illness development (Huang, et al., 2009). Analogous tendencies are realized in rhesus macaques SIV, with Mamu-B*08 and B*17 haplotypes linked with curbing and Mamu-B*01 linked with evolution to AIDS (O'Connor, et al., 2015). Coreceptor genes govern the quantity of expression of co-receptors and co-receptor ligands for example chemokines that can decrease the availability of receptors required for the attachment of HIV virus. The utmost protuberant genome point mutation is the Δ32 removal in CCR5 that revokes the quantitative expression of that particular gene. In its homozygous state, CCR5Δ32 distinctly decreases the danger of infection and, in its heterozygous appearance, decreases the

speed with which an individual progress to AIDS (Singh, et al., 2008). More lately, genome-wide association studies (GWAS) have been employed for a wide-ranging, impartial picture of the human genome and links of HIV-associated ailment. To further examine possible associates, comprehensive studies of the expression of gene profile have been achieved, by means of both microarray studies and straight sequencing of RNA (Freitas, et al., 2014). Many of these studies have shown several single nucleotide polymorphisms (SNPs) that effect susceptibility to infection or the speed of advancement to AIDS (Catano, et al., 2008). Table 2.1 shows the host genetic determinants and SNPs that effect susceptibility to infection or the speed of advancement to AIDS

| **Genetic Locus** | **Genetic Context** | **Biological effect** | **Progression to AIDS** |
|---|---|---|---|
| CCR5 | Homozygous /Heterozygous | Δ32 mutation in CCR5 revokes or lessens CCR5 expression | Deferred progression |
| CCR5 | Homozygous | P1 mutation in promoter for CCR5 | Hastens progression |
| CCR2 | 64I mutation<br>Heterozygous | Unknown | Deferred progression |
| CCL3L1 | Low Copy number | Chemokine ligand for CCR5 | Hastens progression |
| SDF-1 | G801A SNP | Ligand for CXCR4 | Deferred progression |
| HLA-B*35 | Homozygous | CD8 T cells are more constrained | Hastens progression |
| HLA-B*07 | Homozygous | CD8 T cells are more constrained in their recognition of viral epitopes | Hastens progression |
| HLA-B*57 | Homozygous | CD8 T cells are more cross-reactive, able to see larger range of viral epitopes | Deferred progression |
| HLA-B*27 | Homozygous | More probable to present gag epitopes that are structurally limited | Deferred progression |
| IL-10 (-1082 A/G) | Homozygous/Heterozygous | greater CD8+ T cells count | Deferred progression |
| IL-18 -607C>A | Homozygous/Heterozygous | increased IL-18 concentrations | Hastens progression |
| IFNγ-874A/T | Homozygous | Lower levels of CD4+ cells | Hastens progression |
| TGF-B-509TT | Homozygous | Higher levels of TGF-B and CCR5 + CXCR4 | Hastens Progression |

***TABLE 2.1.*** *Genetic determinants and SNPs that effect susceptibility to infection or the speed of advancement to AIDS*

# *Chapter 3*

# *Methodology*

## 3.1 Study design

This study was conducted in collaboration with NACP (National AIDS Control Program) and its referral lab at Pakistan Institute of Medical Sciences (PIMS), Islamabad as per the Letter Of Understanding (LOU) signed between ASAB NUST; NACP and PIMS.

The methodology carried out during the course of the study is depicted in figure 3.1 in the form of a flow diagram.

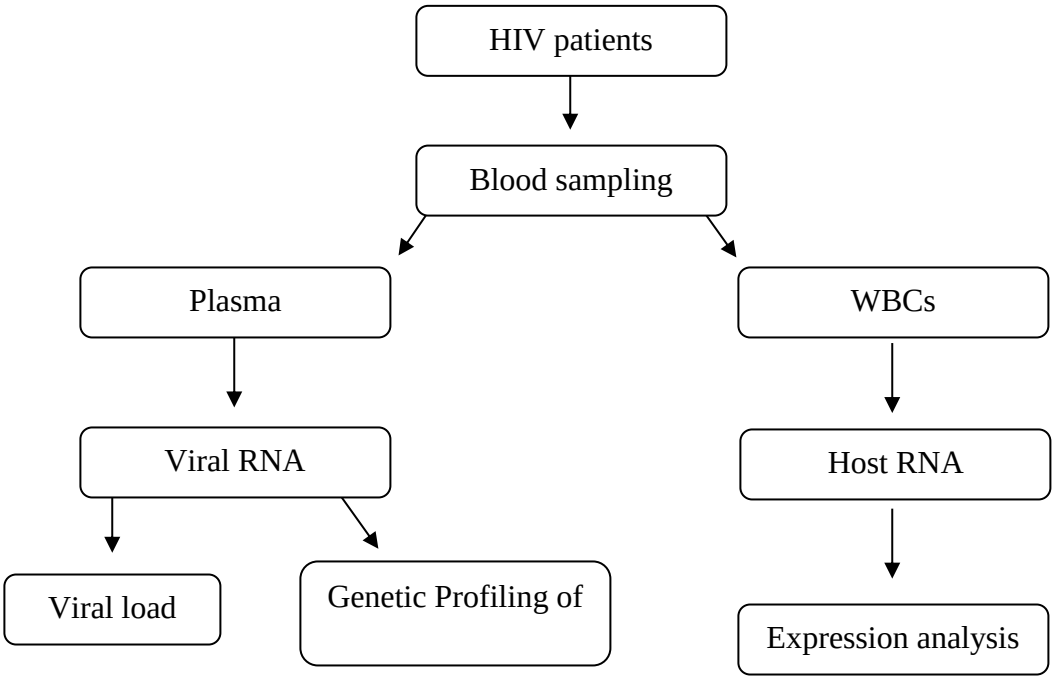



***Figure 3.1****: Schematic flow diagram of the methodology used in this project.*

### 3.1.1 Ethical Statement

All Patients recruited in this study gave a written signed consent after they were well informed about the research being carried out. The study did not commence until written approval was

granted by the Ethical Review Board of Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU) Islamabad, Pakistan and institutional review board (IRB) of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences & Technology (NUST) Islamabad, Pakistan after all the research protocols were thoroughly assessed.

### 3.1.2 HIV blood sampling

Since HIV is a biohazardous agent, all sampling was conducted under strict Biosafety level-2 conditions and all standard precautionary measures were undertaken. A total of 50 HIV-1 positive blood samples (45 men and 5 women), with a mean age of 34±11.8 years were collected at the PIMS referral lab after informed written consent was taken from the patients. The inclusion criteria were only those patients who were either treatment naïve or those who had failed to respond to the antiretroviral treatment (ART). The demographic details including age, sex, viral load, any co-infection and other relevant clinical data was noted down from the patient's card ensuring anonymity at all stages. 40 control samples (10 males and 30 females) with a mean age of 25±10 years were collected from students at Atta-Ur-Rahman School of Applied Biosciences (ASAB), National Institute of Sciences and Technology (NUST), Islamabad after verbal consent and some of the control samples were provided courtesy of Dr. Yasmeen Badshah. It was ensured that the control samples were HIV-negative and had no prior history of HIV/AIDS.

### 3.1.3 RNA extraction (Trizol LS method)

Host RNA was extracted from the HIV-1 positive blood samples and cDNA was synthesized for further QRT-PCR expression analysis at NIH. For this purpose, first 5ml of peripheral venous blood was drawn from each patient and transferred from the sterile syringe into a 5ml

blood K3EDTA vacutainer and centrifuged at 6000 x g for 2 minutes. The upper plasma layer was collected and stored at -80°C for further viral RNA extraction. The remaining blood (2-3ml) was decanted into a 15ml polypropylene conical falcon. A 1X RBC Lysis Buffer was added and the final volume of the mixture was bought up to 15ml. The blood-Lysis buffer mixture was left to stand for 10 minutes at room temperature. Thereafter, the mixture was centrifuged at approximately 600 x g for 10 minutes at room temperature to pellet the PBMCs. The supernatant was discarded and the PBMC pellet was again resuspended in 1ml RBC-Lysis buffer to remove any remaining traces of RBCs. After 5 minutes, the contents were transferred to a fresh 1.5ml Eppendorf tube and centrifuged at 3000 x g for 2 minutes under room temperature. Again, the supernatant was discarded, and the pellet was washed in 1ml of PBS. After another round of centrifugation at 3000 x g for 2 minutes, the pellet was suspended in 250µl of PBS. To this, 750µl of Trizol® LS solution (Thermo Fisher Scientific Inc) was added and the resultant solution was vigorously mixed to ensure complete homogenization. The samples were then stored at -20°C until further processing. The homogenate was then thawed at room temperature and 200µl of chloroform was added. The mixture was thoroughly shaken by hand for a minimum of 15 seconds (no vortex) and then stored at room temperature for 10minutes. The tubes were inverted occasionally to ensure proper mixing. The samples were then centrifuged at 12000 x g for 15 minutes at 4°C. After centrifugation, the mixture was separated in to 3 layers: an upper aqueous layer containing RNA, a white interface containing DNA and a lower reddish phenol-chloroform layer containing proteins. Almost 500 µl of the aqueous layer was carefully removed while taking care not to remove any portion of the underlying interphase and transferred to a new 1.5ml sterile Eppendorf tube. The remaining interphase and organic phase was stored overnight at 4°C for DNA processing the next day. To the aqueous layer, 500 µl of ice-chilled

isopropanol was added and the solution was mixed thoroughly and incubated for 10 minutes at room temperature. Thereafter, the solution was centrifuged at 12000 x g for 10 minutes at 4°C. The RNA forms a gel-like pellet at the bottom of the tube. The supernatant was carefully discarded, and the pellet was resuspended in 1ml of ice-chilled 75% Ethanol. The sample was briefly vortexed and then centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was carefully removed, and remnant ethanol was removed using a pipette. The RNA pellet was air-dried for 10 minutes. The RNA pellet was then Resuspend in 25µL of Nuclease-free (NF) water and incubated in a water-bath at 55°C for 10 to 15 minutes so that the RNA is completely dissolved. The extracted RNA was then quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). A 260/280 of >1.8 suggested pure RNA. The RNA was also run on an agarose gel and Discrete 28S and 18S rRNA bands ensured that that RNA had good integrity.

### 3.1.4 Host cDNA synthesis

For cDNA synthesis, Thermo Scientific™ Maxima™ H Minus Reverse Transcriptase (RT) was used. Briefly, 1 µL (100 pmol) of Random Hexamer, 1 µL of dNTP Mix, 10 mM each (0.5 mM final concentration) and 1-2µg of the extracted RNA were added to a sterile, RNA-free tube placed on ice and made up to 15µl using sterile Nuclease-free water. The mixture was briefly centrifuged and heated at 65°C for 5 minutes. The tube was then immediately chilled on ice for 5 minutes. Thereafter, 4µl of 5X RT-buffer and 1µl of Maxima™ H Minus Reverse Transcriptase (RT) was added to the tube and gently mixed followed by incubation at 25°C for 10 minutes and then at 55°C for 45 minutes. Finally, the reaction was terminated by heating at 85°C for 5 minutes. The cDNA was confirmed using Beta-globin PCR and the cDNA was stored at -20°C for further downstream applications.

### 3.1.5 DNA extraction (Trizol-LS method)

Host DNA was isolated from the interphase and lower organic phase saved from the RNA extraction method at NIH. Briefly, the lower phase was thawed at room temperature and any remnant aqueous layer was removed using another round of centrifugation. Thereafter, 300 µl of 100% Ethanol was added to the tube and inverted several times to ensure complete mixing. The mixture was then incubated at room temperature for 3 minutes and then subsequently centrifuged at 2500 x g for 5 minutes at 4°C. The phenol-ethanol supernatant was discarded carefully, and the DNA pellet was resuspended in 1ml of 0.1M Sodium Citrate in 10% Ethanol solution. The mixture was incubated for 30 minutes at room temperature with occasional mixing. This was followed by centrifugation at 2500 x g for 5 minutes at 4°C to pellet the DNA. The Sodium Citrate washing step was repeated a second time followed by centrifugation at 2500 x g for 5 minutes at 4°C. After this, the pellet was resuspended in 1.5ml of 75% Ethanol and incubated for 20 minutes at room temperature with occasional mixing. This was followed by centrifugation at 2500 x g for 5 minutes at 4°C. The ethanol supernatant was discarded, and the pellet was allowed to air-dry for 10 minutes. Subsequently, the pellet was dissolved in 50-60 µl of 8mM NaOH by pipetting up and down and incubated in a water bath at 55°C for 10-15 minutes. The pH of the solution was adjusted with 10-15 µl of HEPES. The DNA was confirmed on 0.8% agarose gel followed by Beta-globin PCR and stored at -20°C until further processing.

### 3.1.6 Sequence Specific Primer PCR (SSP-PCR) of IL-10, IL-18 &TGF-β

Genotyping of IL-10, IL-18, TGF-β was performed by (sequence specific primer)-SS-PCR analysis also known as allele-specific PCR. It involves two reactions each consisting of a control

forward primer, an allele specific primer and a common reverse primer. The primers used are listed in table 3.1

For IL-18, two reactions were setup each containing the same components except for the allele specific primer. Briefly, a 25 μL PCR reaction was setup on ice consisting of 2.5 μL of 10X PCR buffer, 0.5 μL of 10 mMol dNTP mixture, 1.5 μL of 25 mM MgCl2, 0.5 μL of Either allele specific primer, 0.5 μL of Control Forward Primer and 0.5 μL of common reverse primer, 2 μL of DNA and 0.5 U of Taq polymerase. For the cycling conditions, initial denaturation was carried out at 94°C for 3 minutes followed by 34 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes.

For IL-10 and TGF-β, the recipe was slightly modified. For each allele, two reactions were setup each containing the same components except for the allele specific primer. Briefly, a 20 μL PCR reaction was setup on ice consisting of 2 μL of 10X PCR buffer, 1.5 μL of 10 mMol dNTP mixture, 2 μL of 25 mM MgCl2, 1 μL of either allele specific primer, 1 μL of Control Forward Primer and 1 μL of common reverse primer, 3 μL of DNA and 0.5 U of Taq polymerase. For the cycling conditions, initial denaturation was carried out at 94°C for 5 minutes followed by 35-40 cycles of 94°C for 45 seconds, 55°C for IL-10 primers and 60°C TGF-β primers for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 10 minutes.

All of the PCR products were run on 2% agarose gel for 40minutes at 120V. The gel was visualized under UV in a gel-doc system and bands were analyzed for SNP presence.

| *Table 3.1 Primers designed for reported HIV SNPs* | | | |
|---|---|---|---|
| | **Name** | **Sequence 5'----------3'** | **Product Size** |
| 1 | IL-10F | TATCTGAAGAAGTCCTGATGTC | 318bp |
| 2 | IL-10R | TTCTTTTAGTTGTAAGCTTCTGTG | |
| 3 | IL-10F(A)* | CTACTAAGGCTTCTTTGGGAA | 168bp |
| 4 | IL-10F(G) | CTACTAAGGCTTCTTTGGGAG | |
| 5 | TGF-βF | CTGACCCCAGCTAAGGCATG | 384bp |
| 6 | TGF-βR | AGAGGACCAGGCGGAGAAG | |
| 7 | TGF-βF(C)* | CCTCCTGACCCTTCCATCCC | 197bp |
| 8 | TGF-βF(T) | CCTCCTGACCCTTCCATCCT | |
| 9 | IL-18F | CTTTGCTATCATTCCAGGAA | 301bp |
| 10 | IL-18R | TAACCTCATTCAGGACTTCC | |
| 11 | IL-18F(C)* | GTTGCAGAAAGTGTAAAAATTATTAC | 196bp |
| 12 | IL-18F(A) | GTTGCAGAAAGTGTAAAAATTATTAA | |

### 3.1.7 Restriction fragment length polymorphism (RFLP) PCR of IL-10, IFN-γ & TGF-β

Genes for TGFβ, IFN-γF and IL-10 genes were separately amplified using previously reported primer by Felipe Bonfim Freitas et al., (2015) using regular PCR followed by the identification using the restriction fragment length polymorphism analysis (RFLP) as seen in table 3.2. Briefly, a 25 µL PCR reaction was setup on ice consisting of 2.5 µL of 10X PCR buffer, 0.5 µL

of 10 mMol dNTP mixture, 1.5 µL of 25 mM MgCl2, 0.5 µL of both Forward and Reverse primer for each gene, 2.5 µL of DNA and 0.5 U of Taq polymerase. For all three genes, the cycling conditions were as follows: initial denaturation was carried out at 94°C for 3 minutes followed by 34 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes. All of the PCR products were run on 4% agarose gel for 40minutes at 120V. The gel was visualized under UV in a gel-doc system and bands were analyzed for SNP presence

***Table 3.2 Restriction enzymes, primer sequences, and fragments produced during polymorphism identification.***

| | **Polymorphisms (endonuclease)** | **Sequence 5'----------3'** | **Alleles and fragments(bps)** |
|---|---|---|---|
| 1 | rs2430561 (Hinf I) | INFg F: 5′-GATTTTATTCTTACAACACAAAATCAAGAC-3′ INFg R: 5′-GCAAAGCCACCCCACTATAA-3′ | A: 176 T: 148, 28 |
| 2 | rs1800896 (Mnl I) | IL-10 F: 5′-TCTGAAGAAGTCCTGATGTC-3′ IL-10 R: 5′-CTCTTACCTATCCCTACTTCC-3′ | A: 125, 65 G: 93, 65, 32 |
| 3 | rs1800469 (Dde I) | TGFβ1 F: 5′-GGAGAGCAATTCTTACAGGTG-3′ TGFβ1 R: 5′-TAGGAGAAGGAGGGTCTGTC-3′ | T: 120 C: 74, 46 |

### 3.1.8 Statistical Analysis

The distributions of allele and genotype frequencies for each polymorphism in the case and control groups were done by counting manually and compared using chi-square All statistical analysis was done in GraphPad Prism v7.03

### 3.1.9 Viral RNA extraction and Viral load determination

The viral RNA was isolated from the patient's plasma using **QIAamp viral RNA extraction kit** (Qiagen) and used for viral load quantification using the **artus HI Virus-1 RG RT-PCR Kit** (Qiagen) following the manufacture's recommendations at NIH.

### 3.1.10 Genetic Profiling of HIV variants

The extracted viral RNA was also subjected to the genetic profiling of HIV variants infecting. Genetic profiling of the HIV variants was initially limited to the identification of the infecting genotype. For this purpose, suitable PCR primer sets for the amplification of HIV integrase were selected from literature and were optimized after rigorous testing. The primer sequences are given in table 3.2. Initially 161bp amplicons located within the integrase gene were optimized to confirm presence of viral RNA followed by amplification of a 700bp amplicon that spanned the integrase gene and parts of the RNAse-H for identification of HIV genotype using previously reported primers (T, Nie et al., 2011). One step RT-PCR using **QIAGEN OneStep RT-PCR Kit** was used for the direct amplification of the viral RNA in a single step eliminating the need to synthesize cDNA in a separate step. The PCR amplicons (161bp and 700bp) were purified using the MEGAquick-spin™ Total Fragment DNA Purification Kit following the

manufacturer's recommendations and subsequently sequenced to confirm correct amplification, presence of viral RNA and the infecting HIV viral genotype.

***Table 3.3 Primers designed for reported HIV detection and genotype profiling***

| | **Name** | **Sequence 5'----------3'** | **Product Size** |
|---|---|---|---|
| 1 | HIV-Cf | ACAGTGCAGGGGAAAGAA | 161bp |
| 2 | HIV-R | CCCTTCACCTTTCCAGAG | |
| 3 | HIV-B2f | TGGAGAGCAATGGCTAGTGA | 687bp |
| 4 | HIV-R | CCCTTCACCTTTCCAGAG | |

# *Chapter 4*

# *Results*

# 4.1 SNPs genotyping results using Sequence Specific Primer PCR (SSP-PCR)

## 4.1.1 Genomic DNA (gDNA) extraction validation

The extracted DNA samples of the HIV positive patients were run on 0.8% agarose gel as shown in *Figure 4.1* to confirm the presence of genomic DNA.

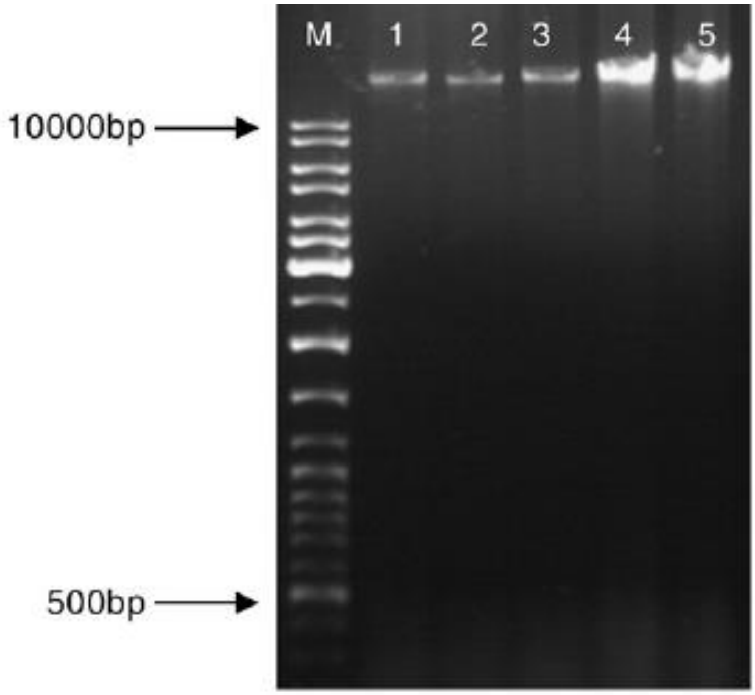



***Figure 4.1.*** *Genomic DNA resolved on 0.8% agarose gel. L: 1Kb plus ladder. Lanes 1-5 show random gDNA samples from HIV-1 patients.*

## 4.1.2 β-globin gene screening for DNA extraction validation

The DNA that was isolated from the HIV positive samples as well as the control samples was checked for amplification and integrity using the β-globin housekeeping gene. A 110bp

fragment established the presence of DNA in both the sample types. The Results for validation of extraction from control samples are shown in *Figure 4.2*.

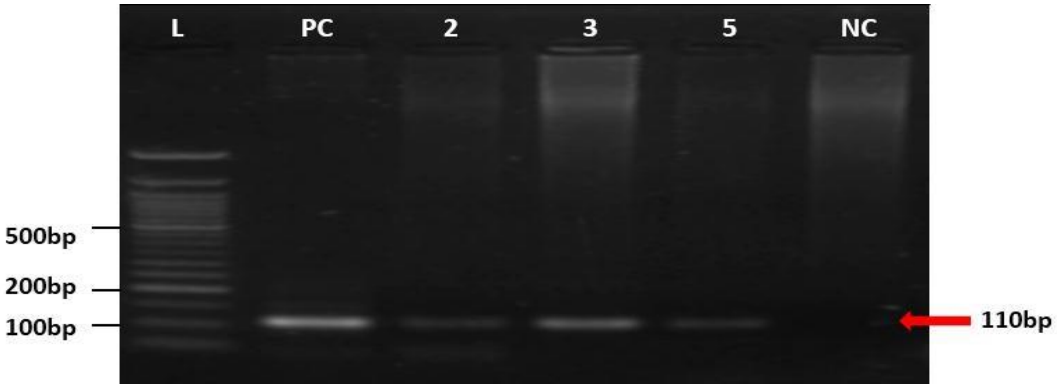



***Figure 4.2**. Amplification of 110bp beta -globin fragment via PCR on control samples (2,3,5). L: 50bp Ladder, PC: Positive Control, NC: Negative control. Visualized on 2% agarose gel.*

Results for the validation of DNA extraction from HIV positive samples using the TRIzol-LS method, are shown in *Figures 4.3*.

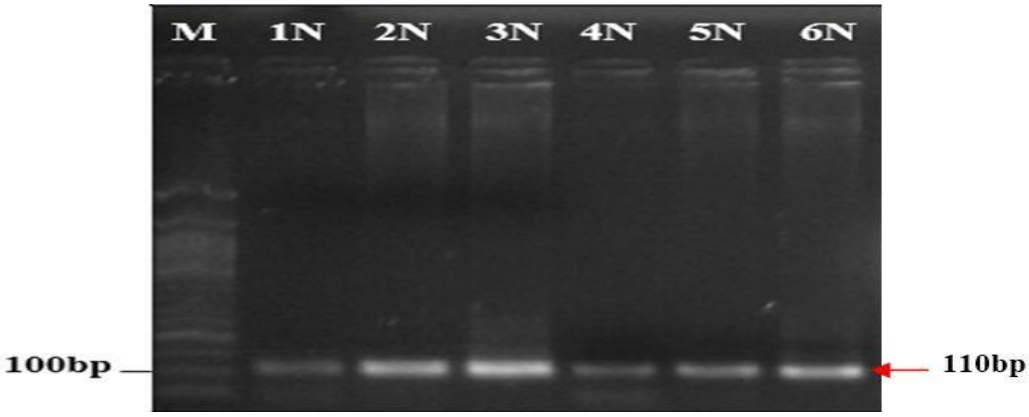



***Figure 4.3.** Beta-globin gene screening in HIV samples. Product size: 110bp. M: 50bp Ladder. HIV samples 1N-6N. Visualized on 2% agarose gel.*

### 4.1.3 Screening for IL-18 (IL-18 −607C>A) polymorphism

#### 4.1.3.1 In Control samples

In all, 40 HIV-1/AIDS patients and an equivalent number of controls were considered for −607C>A IL-18 promoter polymorphism having no prior history of HIV/AIDS. As shown in Figure 4.4, there were A/A, AC and CC genotypes at position −607 within the control samples. The PCR amplicon of homozygous individuals showed a 301 bp DNA segment and those of heterozygous individuals exhibited 196 and 301 bp fragments.

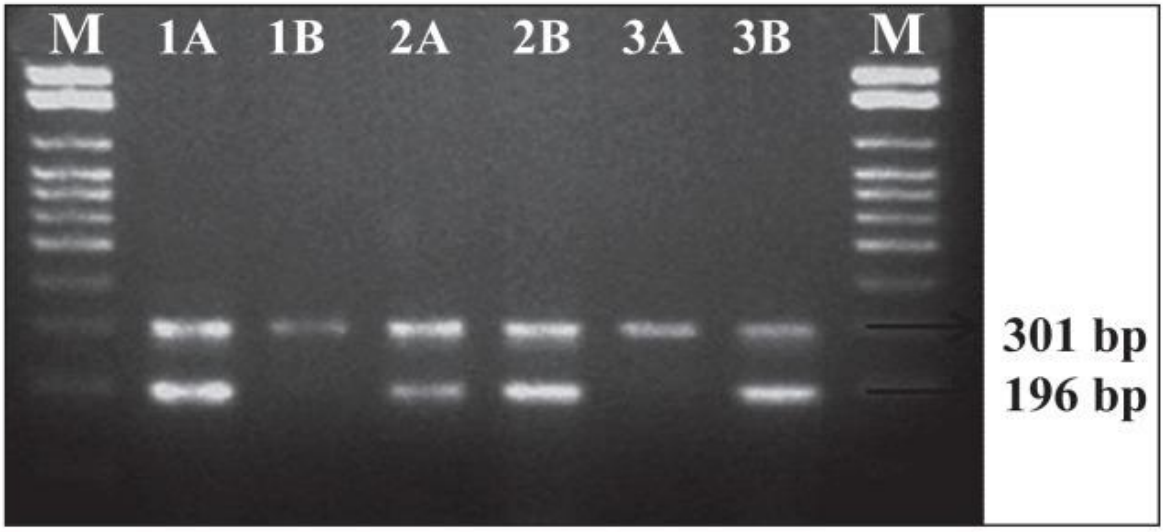



***Figure 4.4.*** *IL-18 −607C>A polymorphism screening in control samples via SSP-PCR. The 301bp PCR amplicon is amplified using the control forward primer and common reverse primer whereas the 197bp amplicon is amplified using the allele specific primer and common reverse primer. L: 100bp Ladder. Lanes 1A and 1B depict CC genotype, 2A and 2B depict AC genotype whereas 3A and 3B depict AA genotype.*

#### 4.1.3.2 In HIV positive samples

In all, only 40 HIV-1 out of 50 HIV patients were considered for −607C>A IL-18 promoter polymorphism owing to the fact that some of the samples had low amounts of DNA due to low

PBMCs. A representative gel picture is shown in *figure 4.5*. The PCR amplicon of homozygous individuals showed a 301 bp DNA segment and those of heterozygous individuals exhibited 196 and 301 bp fragments.

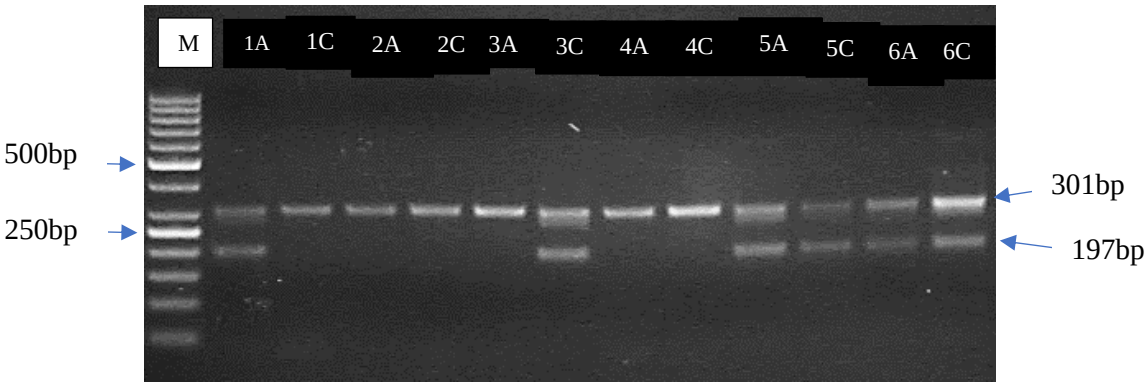



***Figure 4.5.*** *IL-18 −607C>A screening in HIV samples using SSP-PCR. Size of control fragment:301bp and allele specific fragment: 197bp. L: 50bp Ladder. A total of 6 representative samples are shown. A represents A allele sample and C represents C allele sample for the same patient.*

## 4.1.4 Screening for IL-10 (IL-10 −1082A>G) polymorphism

### 4.1.4.1 In Control samples

In all, 40 HIV-1/AIDS patients and an equivalent number of controls were considered for −1082G IL-10 polymorphism having no prior history of HIV/AIDS. As shown in Figure 4.6, there were A/A, AG and GG genotypes at position −1082 within the control samples. The PCR amplicon of homozygous individuals showed a 318 bp DNA segment and those of heterozygous individuals exhibited 168 and 318 bp fragments.

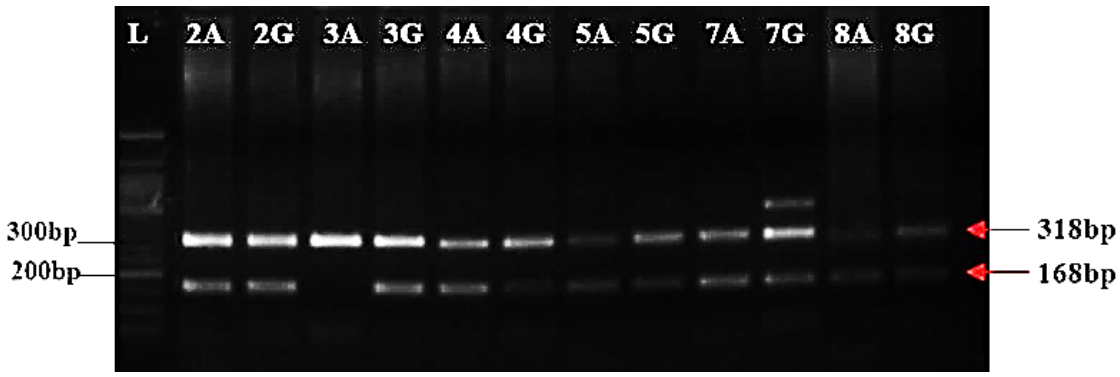



***Figure 4.6.*** *IL-10 polymorphism screening in control samples via SSP-PCR. A represent PCR with A allele specific primer and G represents G allele primer. 2,3,4,5,7,8 are the control sample numbers. Size of control fragment:318bp and allele specific fragment: 168bp. L: 50bp Ladder*

### 4.1.4.2 In HIV positive samples

In all, only 40 HIV-1 out of 50 HIV patients were considered for −1082G IL-10 polymorphism owing to the fact that some of the samples had low amounts of DNA due to low PBMCs. A representative gel picture is shown in *figure 4.7*. The PCR amplicon of homozygous individuals showed a 318 bp DNA segment and those of heterozygous individuals exhibited 168 and 318 bp fragments.

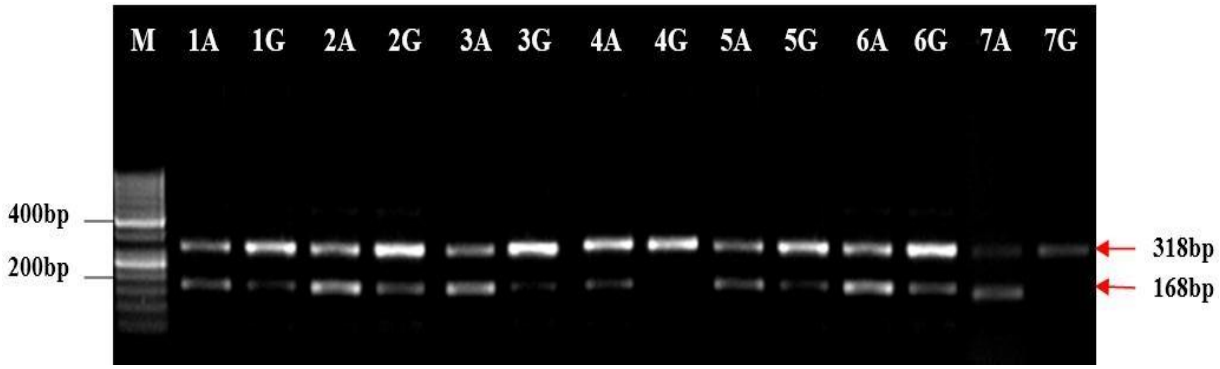



***Figure 4.7.*** *2% agarose gel visualization of IL-10 polymorphism screening in HIV samples. Product size for control fragment and allele specific fragment of IL-10: 318bp and 168bp, respectively. M: 50bp Ladder. Lanes 1A and 1G, 2A and 2G, 3A and 3G, 5A and 5G, 6A and 6G represent heterozygous genotype (AG). Lanes 4A and 4G represent homozygous AA genotype*

## 4.1.5 Screening for TGF-β (-509 C/T) polymorphism in control and HIV samples

In all, 40 HIV-1/AIDS patients and an equivalent number of controls were considered for TGF-β C/T polymorphism having no prior history of HIV/AIDS. As shown in *Figure 4.8*, there were C/C, CT and TT genotypes at position −509 within the control and HIV samples. The PCR amplicon of homozygous individuals showed a 384 bp DNA segment and those of heterozygous individuals exhibited 197 and 384 bp fragments.

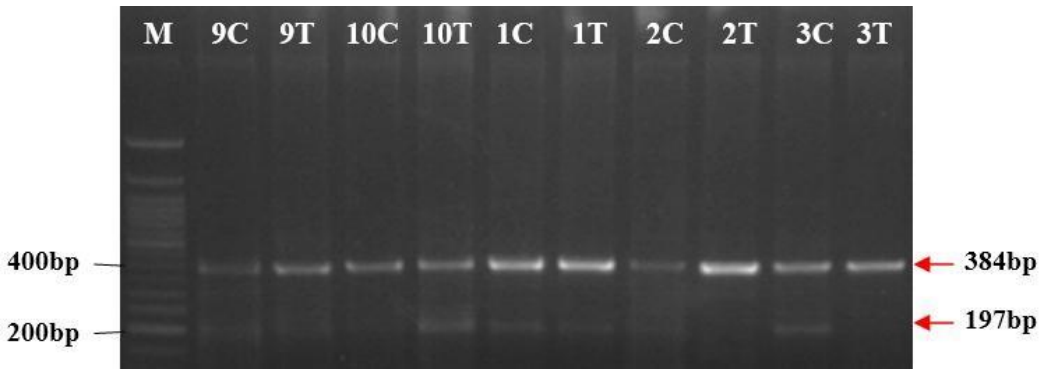



***Figure 4.8.*** *TGF-beta polymorphism screening in HIV and control samples. M: 50bp Ladder. C represent C allele, T represent T allele. Lanes 9C-10C represent 2 HIV samples. Lanes 1C-3T represent control samples.*

## 4.1.6 IL-18 polymorphism statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the IL-18 cytokine SNP being studied. Results of the comparison are given in *Table 4.1.* As seen from the table,

***Table 4.1****. Distribution of genotypic and allelic frequencies of* −607C>A IL-18 promoter polymorphism in the study groups.

| | **HIV** (n=40) | **Control** (n=40) | **p value** |
|---|---|---|---|
| CC | 16 (40%) | 13 (32.5 %) | 0.7418 |
| CA | 22 (55%) | 24 (60%) | |
| AA | 2 (5%) | 3 (7.5%) | |
| **A** | 24 (30.7%) | 38 (37.6%) | 0.3392 |
| C | 54 (69.3%) | 63 (62.4%) | |

The genotypic distribution for -607C/A IL-18 polymorphism is demonstrated in Figure 4.9.

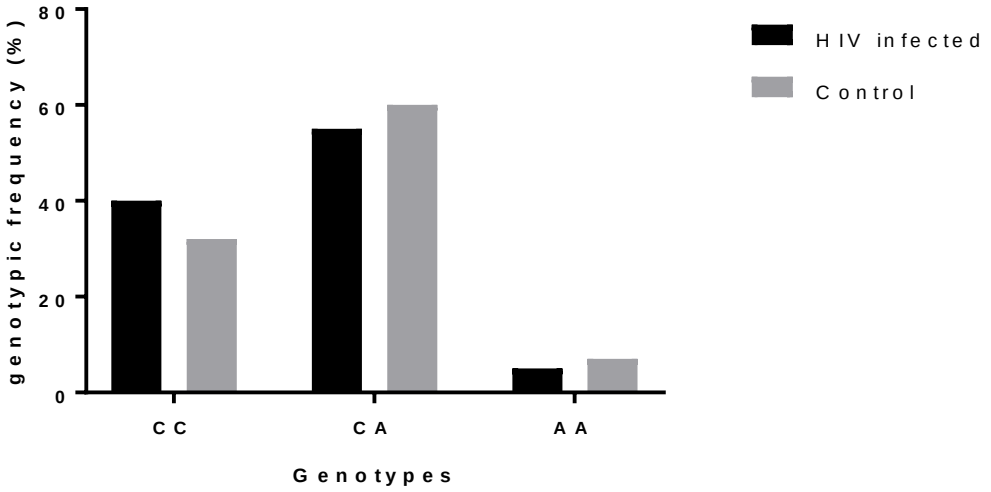



***Figure 4.9.*** *Genotype distribution of IL-18 polymorphism in case and control group*

## 4.1.7 IL-10 polymorphism statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the IL-10 cytokine SNP being studied. Results of the comparison are given in *Table 4.2.* As seen from the table,

***Table 4.2****. Distribution of genotypic and allelic frequencies of IL-10-1082A>G polymorphism in our study groups*

| | **HIV** (n=40) | **Control** (n=40) | **p value** |
|---|---|---|---|
| **AA** | 5 (13.3%) | 4 (10%) | 0.1197 |
| **AG** | 35 (86.7%) | 32 (80%) | |

| | | | |
|---|---|---|---|
| **GG** | 0 (0%) | 4 (10%) | |
| **A** | 45 (56.25%) | 40 (50%) | 0.4283 |
| **G** | 35 (43.75%) | 40 (50%) | |

The genotypic distribution for IL-10-1082A>G polymorphism is demonstrated in Figure 4.10

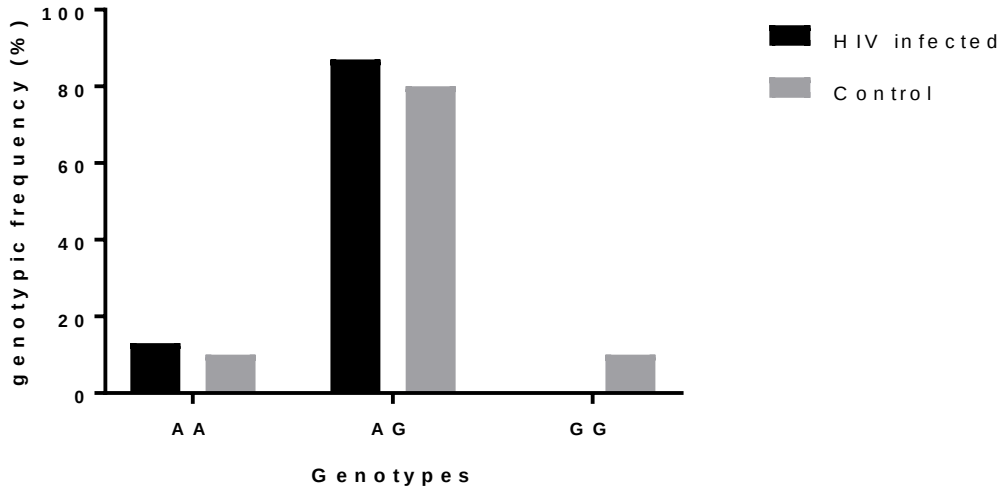



***Figure 4.10.*** *Genotype distribution of IL-10 polymorphism in case and control group*

## 4.1.8 TGF-β polymorphism statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the TGF-β cytokine SNP being studied. Results of the comparison are given in *Table 4.3.*

| ***Table 4.3.*** *Distribution of genotypic and allelic frequencies of TGF-509 C/T polymorphism in our study groups* | | | |
|---|---|---|---|
| | **HIV** (n=40) | **Control** (n=40) | **p value** |
| **CC** | 4 (10%) | 16 (40%) | <0.001 |
| **CT** | 16 (40%) | 24 (60%) | |
| **TT** | 20 (50%) | 0 (0%) | |
| **C** | 24 (30%) | 56 (70%) | <0.001 |
| **T** | 56 (70%) | 24 (30%) | |
| | | | |

The genotypic distribution for TGFB-509 C/T polymorphism is shown in figure 4.11.

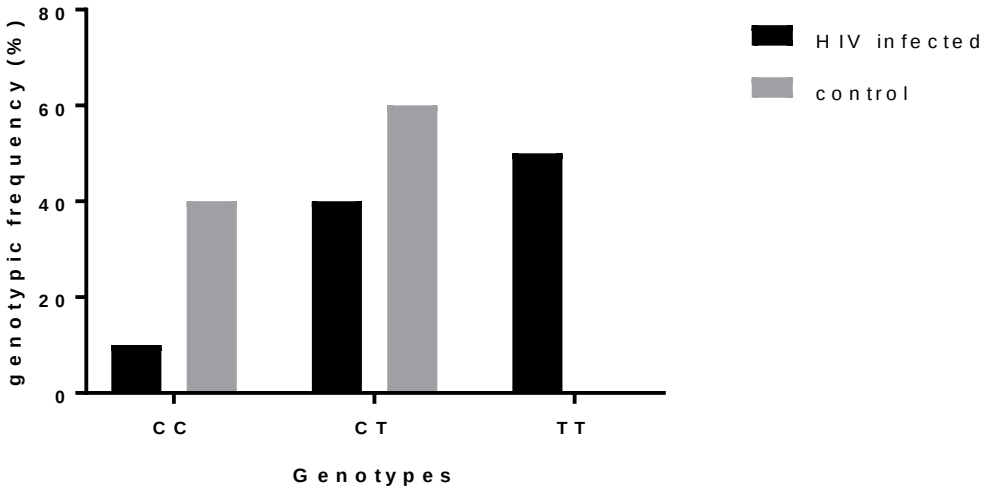



***Figure 4.11.*** *Genotype distribution of TGF-β polymorphism in case and control group*

# 4.2 SNPs genotyping results using Restriction fragment length polymorphism analysis (RFLP)

## 4.2.1 Optimization of IL-10, TGF-β and IFN-γ genes

DNA extracted from control samples was used for the optimization of IL-10, TGF-β and IFN-γ genes using SNP specific primers. Results are shown via representative gel in Figure 4.12.

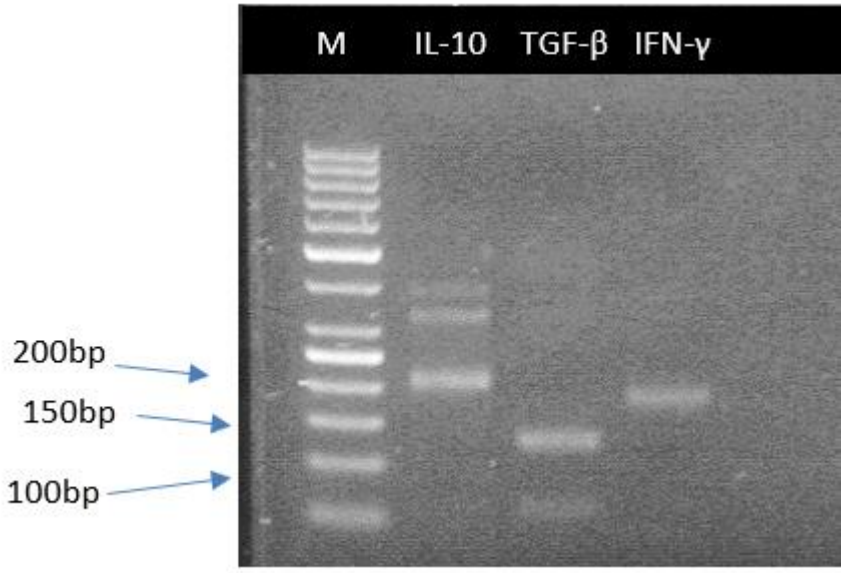



***Figure 4.12.*** *RFLP Primer Optimization using control sample. Product size for allele specific fragment of IL-10: 190bp, TGF-β: 120bp, IFN-γ:176bp, respectively. M: 50bp Ladder.*

## 4.2.2 Screening for IL-10 (IL-10 −1082A>G) polymorphism in HIV samples

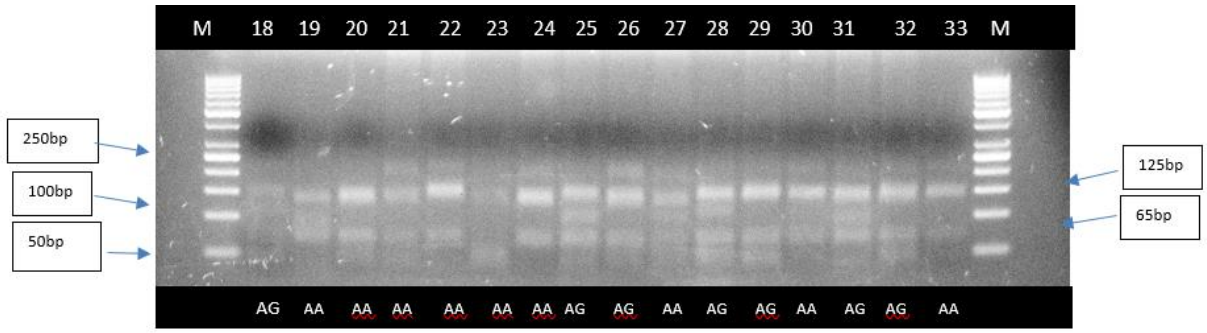



***Figure 4.13.*** *4% agarose gel visualization of IL-10 RFLP in HIV samples. Product size for AA genotype of IL-10: 125bp and 65bp, respectively. Product size for AG genotype of IL-10: 125bp, 65bp 93bp & 32bp, respectively. Product size for GG genotype of IL-10: 93bp, 65bp & 32bp respectively M: 50bp Ladder.*

## 4.2.3 Screening for IFN-γ (+874 T/A) polymorphism in HIV samples

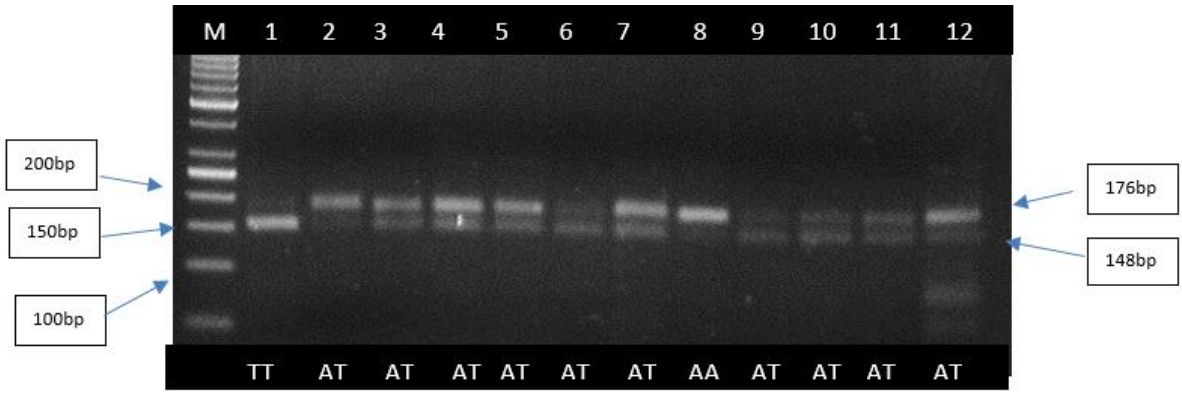



***Figure 4.14.*** *4% agarose gel visualization of IFN-γ RFLP in HIV samples. Product size for AA genotype of IFN-γ: 176bp respectively. Product size for AT genotype of IFN-γ: 176bp, 148bp & 93bp & 28bp, respectively. Product size for TT genotype of IFN-γ: 148bp & 32bp respectively M: 50bp Ladder.*

### 4.2.4 Screening for TGF-β (-509 C/T) polymorphism in HIV samples

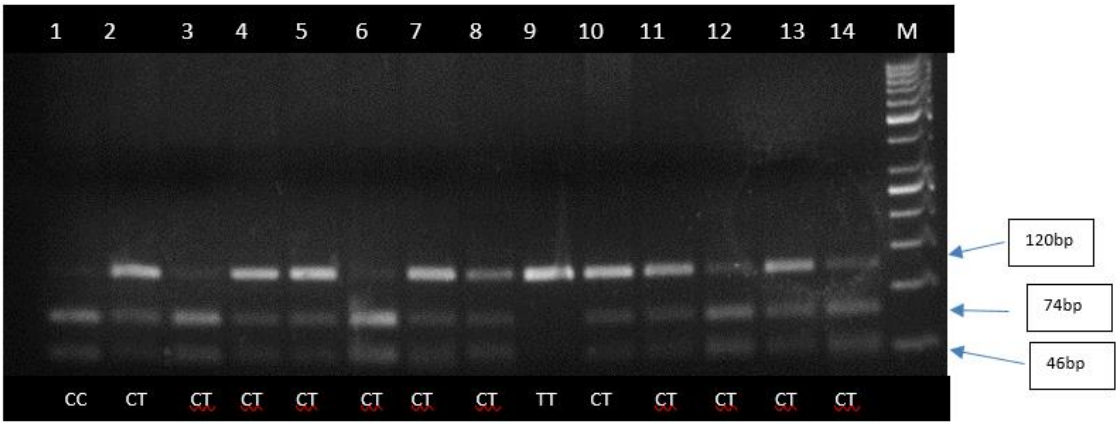



***Figure 4.15.*** *4% agarose gel visualization of TGF-β RFLP in HIV samples. Product size for TT genotype of TGF-β: 120bp respectively. Product size for CT genotype of TGF-β: 120bp, 74bp & 46bp, respectively. Product size for CC genotype of TGF-β: 74bp & 46bp respectively M: 50bp Ladder.*

### 4.2.5 IL-10 RFLP statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the IL-10 cytokine SNP being studied. Results of the comparison are given in *Table 4.4.* As seen from the table,

***Table 4.4****. Distribution of genotypic and allelic frequencies of IL-10-1082A>G polymorphism in our study groups using RFLP*

| | **HIV** (n=30) | **Control** (n=40) | **p value** |
|---|---|---|---|
| **AA** | 16 (53.3%) | 4 (10%) | 0.0002 |
| **AG** | 14 (46.7%) | 32 (80%) | |
| **GG** | 0 (0%) | 4 (10%) | |
| **A** | 46 (76.7%) | 40 (50%) | 0.0013 |
| **G** | 14 (23.3%) | 40 (50%) | |

### 4.2.6 IFN-γ RFLP statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the IFN-γ cytokine SNP being studied. Results of the comparison are given in *Table 4.5.* As seen from the table,

***Table 4.5**. Distribution of genotypic and allelic frequencies of IFN-γ (+874 T/A) polymorphism in our study groups using RFLP*

| | **HIV** (n=40) | **Control** (n=40) | **p value** |
|---|---|---|---|
| **TT** | 2 (5%) | 4 (10%) | 0.3641 |
| **AT** | 26 (65%) | 20 (50%) | |
| **AA** | 12 (30%) | 16 (40%) | |
| **A** | 50 (62.5%) | 52 (65%) | 0.7422 |
| **T** | 30 (37.5%) | 28 (30%) | |

### 4.2.7 TGF-β RFLP statistical analysis

The two studied groups, HIV infected patients and control group, were compared for their allelic and genotypic distribution of the TGF-β cytokine SNP being studied. Results of the comparison are given in *Table 4.6.*

***Table 4.6.*** *Distribution of genotypic and allelic frequencies of TGF-509 C/T polymorphism in our study groups using RFLP*

| | **HIV** (n=40) | **Control** (n=40) | **p value** |
|---|---|---|---|
| **CC** | 3 (7.5%) | 16 (40%) | 0.0015 |
| **CT** | 35 (87.5%) | 24 (60%) | |
| **TT** | 2 (5%) | 0 (0%) | |
| **C** | 41 (51.2%) | 56 (70%) | 0.0152 |
| **T** | 39 (48.8%) | 24 (30%) | |

## 4.3 HIV genetic profiling

In this study, we sought to confirm the presence of the viral RNA in the plasma samples using the 161base pair amplicon and further sequencing was carried out using the 700bp amplicon. Figure 4.13 shows the representative gel for the amplicons which was optimized using One-Step RT PCR and Figure 4.17 shows a representative sequence alignment for the 700bp amplicon.

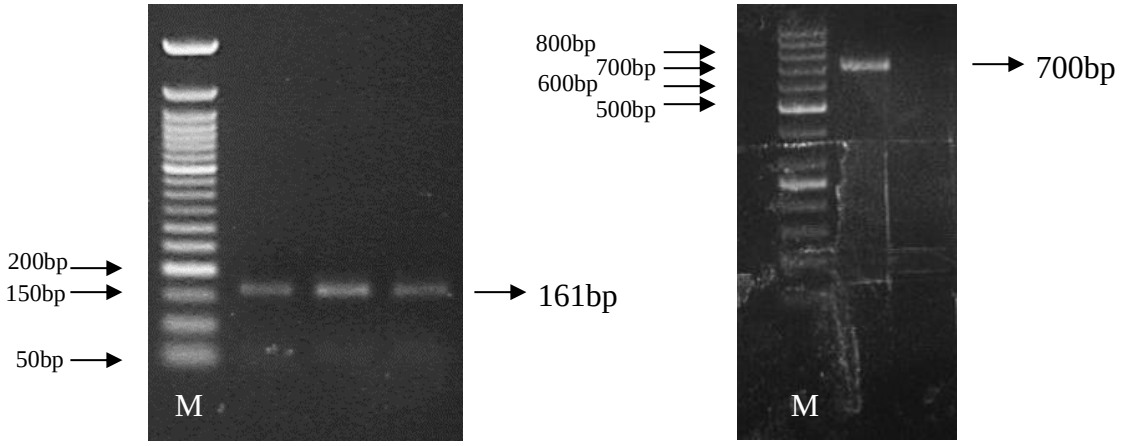



***Figure 4.16**: Representative gel of the optimization of one step RT-PCR for 161bp (A) and 700bp (B) amplicons for the confirmation of viral RNA presence and HIV genotyping respectively. The amplicons are sized against 50bp DNA ladder (M).*

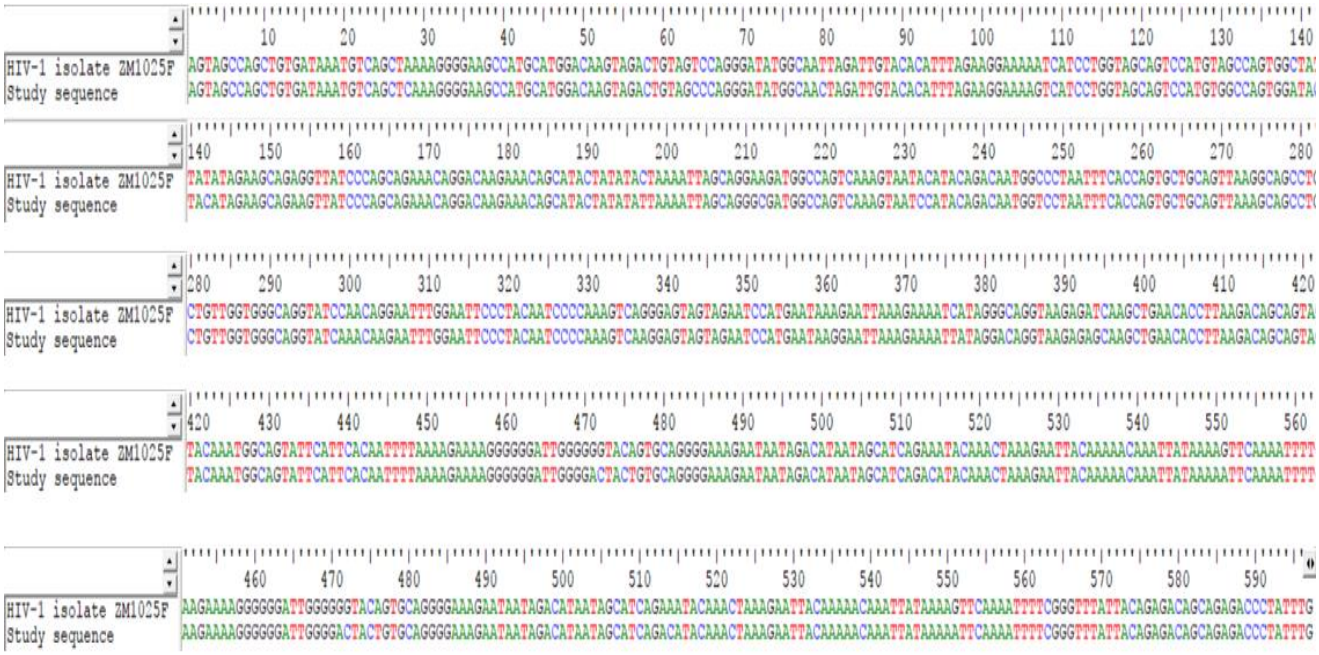



***Figure 4.17*** *Alignment of a representative 700bp HIV-1 pole gene region (Query) to an HIV-1 isolate ZM1025F from Zambia (Subject) with 97% homology. The sequence has been clipped to eliminate sequencing errors.*

# *Chapter 5*

# *Discussion*

The global aim to curb HIV/AIDS by 2030 requires the underpinning of multifaceted mechanisms and the array of factors that have a key role in this disease. It has been seen that most of the polymorphisms are located within the introns and promoter regions thereby regulating the expression of certain cytokine genes and influencing the binding capacity of transcriptional factors. The SNPs chosen in this study were selected because of their potential role in determining the pathogenicity of HIV infection as well as the response to treatment and control of infection i.e. progression to AIDS. In All, the genetic and allelic distribution of SNPs within the study mostly indicated non-significant results owing to the limited number of samples. Freitas et al. had studied the associations of 5 SNPs with HIV infection and pathogenicity out of which 3 exhibited significant results (Freitas, et al., 2014). One of these polymorphisms is -1082 A/G in IL-10 which in theory lowers CD8+ T cells count. IL-10 is a macrophage growth inhibiting cytokine also constraining HIV replication in these cells reported by Kollman et al. (1996) Different IL-10 genetic variants have exhibited various association to AIDS progression such as lower IL-10 serum levels have been linked to a higher risk of rapid progression to AIDS while at the same time conferring protection in other groups such as a study carried out by Winkler et al. showed that a transition from A>C at position -592 within the IL-10 promoter was linked to a slower progression to AIDS. Naicker et al. found that the AA and AG genotypes at position 1082 conferred protection by enhancing the immune response of HIV specific CD8+ T-cells. (Naicker, et al., 2012). In the present study, an equal number of AG and AA genotypes indicating that they did not differ significantly between the case and control population (P=0.1197) as well as the allele frequency of A and G was non-significant occurring at almost equal frequencies (P=0.4283). Most of this can be accounted for the low sample

number used in this study due to time and resources constraint. Surprisingly, the GG genotypes which is associated with a rapid progression to AIDS was found within the control population.

The second polymorphism under investigation was the IL-18 -607C>A polymorphism. IL-18 is a proinflammatory cytokine that augments HIV-1 viral replication. SNPs within the promoter region of IL-18 lead to changes in the quantitative expression of this cytokines as well as alter the transcriptional activity of this gene. 607C/A and C/C genotypes have been associated with a higher level of IL-18 as well as rapid progression to AIDS as shown in a study by Sobti et al involving 500 HIV/AIDS infected patients from North India. (Sobti, et al., 2011). The AA genotypes corresponds to a lower level of IL-18. The C to A transition has been shown to disrupt the binding of CREB protein (B, et al., 1992). An investigation carried out by Segat et al. (2006) in Brazilian children infected with HIV also related this polymorphism to differential HIV susceptibility. In this study, similar to IL-10, the CC and CA genotypes did not differ significantly between the case and control population group (P= 0.7418) neither did the C and A allele frequency (P=0.3392). The CC and CA genotypes were found in higher number in both the control as well as control population which have been associated with a rapid progression to AIDS.A larger sample size is needed to justify this claim if whether these genotypes prevail amongst the general Pakistani population too.

The last SNP was studied within the TGF-βgene. TGF-β is recognized as a CTLA-4 stimulant and its expression correlated to progression markers, and IL-2 inhibition (G. C. Blobe, 2000). In HIV infected people, CTLA-4 positive T-regulatory cells are amplified triggering virus related immune dysfunction (I, 2005). The study suggested T allele's likely association with a greater HIV-1 susceptibility which can be partially be described by TGF-β's linkage to increased expression of the HIV coreceptors CCR5 and CXCR4 vital for viral entry (J. L. Riley, 2000). In

this study, the TT genotype differed significantly between the control and case group (P=0.001) with a higher TT genotype in HIV patients being linked to a higher level of TGF-β. Since all of our patients were treatment Naïve or failure cases, the TT genotype might be found more frequently in HIV patients with a high viral load and thus increase susceptibility.

The sequencing results confirmed the HIV-1 isolates and these results will be beneficial in future sequence analysis such as drug resistance mutation analysis, genotyping, subtyping, phylogenomics, proteomics and other analysis.

# *Chapter 6*

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