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ASSOCIATION OF POL GENE POLYMORPHISM WITH ANTIRETROVIRAL DRUG RESISTANCE IN HIV-I INFECTED PATIENTS FROM LAHORE, PAKISTAN

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ABSTRACT

Background: *Genetic variability in HIV-1 poses significant obstacles to treatment efficacy, particularly in regions like Lahore, Pakistan. This study aimed to characterize genetic diversity in HIV-1 pol gene among high-risk groups in Lahore.*

Methodology: *Thirty-five plasma samples were collected. After viral RNA extraction, pol gene amplification was done using thermocycler. Sanger sequencing was done to detect genetic variability involved in drug resistance. Bioinformatics analysis was performed to build phylogenetic tree and to do assessment of drug resistance in protease (PR), integrase (IN) and reverse transcriptase (RT) regions of HIV-1 pol gene.*

Results: *There were genetic similarities with HIV-1 separates from Pakistan and South Africa. Phylogenetic analysis classified sequences into sub-subtypes 02_AG, subtype A, and subtype G. Subtype 02_AG predominated, representing 44% prevalence in Lahore. We found significant substitutions along PR regions at 113V, K14R, L19P, K20I, N37D, L63S, H69K, M36I, L89M. Resistance mutations to nucleoside reverse transcriptase inhibitor (NRTI) at regions S68G, D67DN and V75VM were found. Regarding non-nucleoside-analog-reverse-transcriptase-inhibitors, resistance was found at E138A and V106I. Other substitutions that were detected through complete length of RT area were K32Q, V35T, E36I, K49R, I135V, D123E, S162A, T165I, Q174K, D177E, K173T, I178L, T200A, Q207E, P243A, V245Q, R356K, S322T, E291D, P294T, A272P, T286A, V292I, I293V, I326V, G335D, M357R, G359S, K366Q, T369A, E370G, A371V, I375V, T377I, K390S, K395R, A400T, T403M, E432D, R461K, D471E, K476R,Q480H, H483Y, K512R, L491S, L517I, S519N, Q524K, K527E, A534S , A554N, E529D.There was only one E157Q mutation in the Integrase strand transfer inhibitors (INSTI) accessory region.*

Conclusion: *There is a low prevalence of drug-resistance-associated mutations in the isolated strains. Protease inhibitors in combination with reverse transcriptase inhibitors may be used to treat these patients in the future.*

INTRODUCTION

Human immunodeficiency virus (HIV) is the cause of acquired immune deficiency syndrome (AIDS), this virus is categorized in the genus Lentivirus within the family Retroviridae. It is an RNA virus with a genome size of 9.2 kb and it encodes in an order of 5'-gag-pol-vif-vpr-tat-rev-vpu-env-3' for the structural and regulatory proteins. HIV type-1 (HIV-1) and HIV type-2 (HIV-2) are the two forms of the virus. Compared to HIV-2, which is found only in some parts of South Africa, HIV-1 is more prevalent globally [1]. The HIV-1

genome has 9 genes which are involved in coding fifteen viral proteins. The Capsid, Matrix and Nucleocapsid are coded by *Gag* gene, while the *Pol* gene encodes three enzymatic proteins reverse transcriptase (RT), integrase (IN), and protease (PR). The viral envelope proteins gp41 and gp120 are coded by *env* gene. Other genes such as *rev* & *tat* encode for regulatory proteins and *vpr*, *vpu*, *nef*, *vif* encode for accessory proteins. Moreover, the viral genome's core also encloses the proteins *vpx* and *vpr* [2].

Replication of the virus depends upon the viral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR), which are all encoded by HIV-1 pol gene. The presence of alterations in the areas of proteins encoded by the pol gene is correlated with antiretroviral treatment resistance and subtype differentiation [3]. Incapability of Reverse transcriptase to perform proofreading functions results in a high rate of genomic mutation and recombination in HIV-1. Thus, with the passage of time, viral variants such as drug-resistant and escape mutants have appeared which are responsible for the failure of vaccination and antiviral drug treatment [4]. In fact, the HIV-1 virus has evolved into a variety of genetic recombinant forms, including circulating and unique recombinant forms (CRFs and URFs). Around 90 CRFs are now distributed worldwide, according to the Los Alamos HIV Database [5]. HIV-1 is divided into three groups: M (Main), N (non-M/non-O), and O (Outlier) which originated in 1920, 1931, and 1963 respectively. Group O cases have been found worldwide due to trans-boundary travel. From gorillas came another group called P (pending). In group M, HIV-1 strains account for 90% of diverse cases having variety of genetic recombinant forms, including CRFs and URFs. While, Group N is phylogenetically equidistant from group M and O and is present only in Cameroon and Gabon [6].

HIV-1 is mainly transmitted sexually. Other modes of transmission are contact with infected blood, reusing and sharing of infected needles, through infected pregnant mothers to fetus, and by needle prick injury to healthcare personnel [7]. Additionally, 2% of new HIV infections in the US in 2018 were presented in transgenders [8].

Antiretroviral therapy is used to treat HIV-1 infection. These antiretroviral medications obstruct several phases of virus replication. Many classes of drugs are currently in practice. Highly active antiretroviral therapy (HAART), is the current standard of treatment for HIV-1 infection, and it involves the administration of three medicines from two separate antiretroviral medication classes. Due to improvements in HIV-1 patient care and treatment, the mortality and morbidity linked to HIV-1 infection have been significantly reduced [9].

Cross resistance between some drugs causes drug resistance in HIV-1 infection, which makes choosing the right treatment regimen more difficult. Hence, testing for drug resistance is crucial for managing patients and maximizing the efficacy of therapy. Data from surveillance studies revealed that about 10% of newly acquired HIV-1 infections are resistant to drugs. Resistant strains are spread by people who have acquired resistance strains *de novo* [10].

AIDS has become a major health problem in Pakistan and has detrimental consequences on other emerging nations as well due to current socio-economic situations [11]. The number of clinical cases of AIDS has climbed to 0.1650 million since its first case which was reported in 1987. The province of Punjab has the highest number of HIV infections (75,000 cases), according to the National AIDS Control Program [12]. Thus, the province of Punjab was an ideal region to study HIV-1 genetic variations and any consequent resistance-associated mutations. For a better understanding of viral epidemiology and drug resistance associated-genomic features, data regarding the presence of various variants, subtypes, sub-subtypes, and CRFs in a few other Asian countries is sufficient. [13]. However, there have only been a few HIV-related researches carried out in Pakistan providing information on the circulating subtypes and resistance-related variants among the common Human immunodeficiency viruses [11,14]

Due to poor financial circumstances and social stigma, the majority of HIV-1 patients in our country are hesitant to visit HIV treatment facilities on a regular basis [11]. Due to this, there is a great likelihood that the course of therapy is interrupted, resulting in mutations. Consequently, phylogenomic and resistance-associated features of the many subtypes of HIV allows for the development of essential and modified therapies for the treatment and control of HIV [15]. Therefore, the aim of the current study was to evaluate the molecular epidemiology, and drug resistance pattern in HIV-1 infected individuals of Pakistan. To achieve this goal, we

investigated the genetic variability and mutations in HIV-1 protease, reverse transcriptase, and integrase gene among high-risk groups in Lahore.

Methodology

Study setting and participants

Thirty-five plasma samples were obtained from the subjects having age >18 years and HIV-related behaviors including history of a drug injection, prostitution, and homosexuality. Samples were collected from Primary and Secondary Healthcare Department HIV treatment centers, especially from the Punjab AIDS Control Programme (PACP) in Lahore. This study was conducted at the laboratory of Influenza within the Department of Microbiology at the University of Veterinary and Animal Sciences in Lahore, Pakistan.

Sample size calculation

The sample size of 35 subjects was calculated using the 5% level of significance and 95% power of the test. The expected prevalence of HIV-infected people was taken into account, with a prevalence rate of 2% [8]. The given formula was used to analyze the sample size:

$$n = \frac{Z^2{}_{1-\alpha} P(1-P)}{d^2}$$

Where,

$Z_{1-\alpha}$= Confidence level = 95% = 1.96
d = Absolute precision = 5%
P = Prevalence = 2%
n = sample size = 35

Ethical approval

The research project followed all recommended rules for the use of human biological samples. The study was conducted after receiving approval from Institutional Review Committee (IRC) at the University of Lahore with Reference number IMBB/BBBC/23/140. Written informed consent was obtained by the subjects at the time of recruitment.

Plasma samples screening

Plasma samples collected from study participants underwent initial screening using the HIV-1 Ag/Ab Combo test (Alere Determine™ HIV US) in agreement with the manufacturer's instructions. This assay was selected for its ability to identify both HIV-1 antibodies and antigen in the samples. Interpretation of test results was conducted rendering to the producer's guidelines.

Viral RNA extraction

Following the early transmission, the viral RNA was removed from antigen positive samples using the FavorPrep TM Viral Nucleic Acid Extraction Kit I (300 preps) (Lot. # CA70920709) giving to the producer's guidelines. The RNA removal process from plasma samples involved several sequential steps to isolate viral RNA suitable for subsequent analysis. First, a lysis tube was prepared by combining 25 µL of protease with 200 µL of plasma. Following this, 6.5 µL of carrier RNA and 200 µL of Buffer AL were additional to mixture, which was then vigorously vortexed to ensure thorough mixing. To facilitate viral lysis and RNA release the prepared mixture was heated to 56ºC for 15 minutes. After incubation, the lysate was briefly centrifuged to pellet debris, and then 250 µL of 96–100% ethanol was added to the supernatant. The ethanol-treated lysate was left to stand for five minutes before another brief centrifugation step. The clarified lysate was carefully transmitted into a QIAamp Mini Column and centrifuged to bind RNA to the column matrix. The column was then eroded successively with 500 µL of Buffer AW1, followed by 500 µL of Buffer AW2, to remove impurities and contaminants. Subsequently, 500 µL of 96–100% ethanol was applied to the column to further wash away residual impurities.

To eliminate any residual wash buffer and ethanol washed column was then transmitted to a fresh collection tube and centrifuged at an extreme quickness for three minutes (2000 x g). To dry column membrane, it was nurtured at 56°C for three minutes. Finally, to elute the isolated RNA from the column matrix, 50 µL of Buffer AVE was added directly onto the column membrane, followed by centrifugation. The eluted RNA was collected in the provided collection tube and stored under -20°C for subsequent analysis.

cDNA synthesis from RNA

Following the extraction of RNA, cDNA synthesis was performed using a standardized protocol. The initial reaction volume was 12 µL, which was achieved by adding 5 µL of extracted RNA, 1 µL of Random Hexamer Primer, and 6 µL of nuclease-free water into a 0.2 ml tube. The required reaction conditions were then provided by adding 4 µL of 5X reaction buffer. Furthermore, 1 µL of RiboLock RNase Inhibitor (20U/µL) was added to stop RNA degradation. Furthermore, 2 µL of a mix containing mMdNTPs (modified nucleotides) was added to provide the necessary nucleotides for cDNA synthesis. To catalyze the reverse transcription reaction, 1 µL of RevertAid M-MuLV Reverse Transcriptase (200U/µL) was introduced into the mixture. The components were gently mixed and briefly centrifuged to ensure proper mixing before being placed into a thermocycler programmed with specific conditions optimized for cDNA synthesis. Cycling conditions for annealing, polymerization and enzyme inactivation were 25°C for 5 minutes, 42°C for 60 minutes and 70°C for 5 minutes, respectively. The cDNA was stored at -20°C for subsequent use in genetic analysis.

Touchdown PCR and RT-PCR

Touchdown PCR was conducted to optimize the amplification of the HIV-1 pol (pol) gene, which spans 1084 base pairs. Using various volumes of nuclease-free water and cDNA, we conducted optimization experiments within a temperature range of 62-52°C. The Touchdown PCR assay for optimizing the HIV-1 pol gene involved a series of temperature-specific steps which are illustrated in Table 1.

Table 1: Cycling conditions for Touchdown PCR assay for HIV1 pol gene optimization

Phases	Temperature (°C)	Period	Rotations
cDNA synthesis	25	5 minutes	
Initial denaturation	94	2 minutes	1x
Denaturation	94	15 seconds	35x
Annealing	62-52	30 seconds	
Extension	68	2 minutes	
Final extension	68	10 minutes	1x
Hold	4	∞	

The HIV-1 pol-gene (1084 bp) was improved by (RT-PCR) reverse-transcriptase-polymerase-chain-reaction using Platinum® Taq DNA Polymerase (Invitrogen, CA, Carlsbad) and SuperScript® III using a methodology outlined by Ou *et al.,* [16]. The RT-PCR assay for amplifying the HIV-1 pol gene involved a series of temperature-specific steps performed over multiple cycles which are illustrated in Table 2.

Table 2: Cycling conditions for RT-PCR assay for HIV-1 pol gene amplification

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	94	4 minutes	1x
Denaturation	94	15 seconds	35x
Annealing	57.6	30 seconds	
Extension	68	2 minutes	
Final extension	68	10 minutes	1x
Hold	4	∞	

Gel electrophoresis

0.5g of agarose from iNtRON BIOTECHNOLOGY® (Cat. 32032) was precisely balanced and diluted in 50 mL of Thermo-Scientific 1X TAE buffer (Lot. No. 00441684) in order to prepare a 1% agarose gel. After heating the mixture for two minutes in the microwave to make sure ideal dissolving, it was cooled. To the mixture, three µL of ethidium bromide was added. After the prepared agarose solution was poured onto a gel casting tray, wells for sample loading were carefully created by inserting a comb. The agarose was allowed to set before the combs were carefully taken out. Three µL of each sample and two µL of the gene ruler were properly pipetted into each well to load the gel. Next, the gel was operated for 50 minutes at 85 volts. Following electrophoresis, the separated DNA bands stained with ethidium bromide were visualized on the gel utilizing either a UV transilluminator or a Gel Documentary system (Major Sciences).

Sanger sequencing

Following amplification of HIV-1 pol gene, PCR products underwent purification using Promega's Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, Wisconsin, USA) to eliminate excess primers, nucleotides, and other impurities. Subsequently, bidirectional sequencing was completed using ABI (Applied Biosystems) Prism 310 Genetic Analyzer. For sequencing, both forward and reverse primers were utilized. The forward primer used was PRT-F2 (CTTTARCTTCCCTCARATCACTCT), located between positions 2243 and 2266 based on the HXB2 reference sequence. The reverse primer employed was RT-R2 (CTTCTGTATGTCATTGACAGTCC), situated between positions 3326 and 3304 on the HXB2 reference sequence.

Subtyping classification and phylogenetic analysis

The REGA HIV-1 Subtyping Tool (Version 3.0) was recycled to evaluate the subtype determination of the sequences under investigation, and online jumping profile Hidden Markov Model (jpHMM) was used to corroborate the results. The resulting regions were then aligned with reference sequences using BioEdit software's ClustalW technique. A tree with the general temporal reversible, gamma distribution, and invariant nucleotide sites was built using the neighbor-joining technique and the Kimura-2 parameter model in order to comprehend the phylogenetic connections. Using the MEGA 7.0 programme, 1000 bootstrap replications were used for these computations.

Approximation of antiretroviral drug resistance

Estimation of antiretroviral drug resistance began with an assessment of sequence quality using Calibrated Population Resistance (CPR) tool version 6.0, accessible at (http://cpr.stanford.edu/cpr.cgi). This initial step involved evaluating the reliability and accuracy of the obtained genetic sequences, ensuring their suitability for subsequent drug resistance analysis. Subsequently, drug resistance mutations were investigated using a genotyping resistance interpretation algorithm that integrates data on NNRTI (non-nucleoside reverse transcriptase inhibitor), protease inhibitor (PI), NRTI (nucleoside reverse transcriptase inhibitor) and Integrase (IN) drugs obtainable in the Stanford HIV database. This algorithm compares the observed genetic sequences against a curated database of known drug resistance mutations associated with specific antiretroviral therapies.

Statistical analysis

The study employed IBM SPSS Statistics (version 21.0) to analyze and explore potential correlations between demographic parameters, categorical variables, and the HIV-1 status (positive or negative) of the participants. A statistically significant level of $p < 0.05$ was considered to indicate meaningful relationships between the variables being analyzed. Using SPSS, chi-square test was utilized to assess associations between demographic factors (such as age, occupation and gender) and HIV-1 infection status.

Results

The demographic and clinical characteristics

The Alere HIV Combo test found 28 samples that tested positive for HIV-1 during the first screening. This represented 80% of the samples, with a 0.593–0.932 95% confidence interval. District Lahore had the highest incidence rate (93%; 95% CI: 0.680-0.998), followed by Gujranwala (75%; 95% CI: 0.349-0.968), Faisalabad (100%; 95% CI: 0.398-1.000), and Sargodha (62%; 95% CI: 0.245-0.915).

Those who had previously used injectable drugs had a considerably increased chance of contracting HIV-1 (68.08%; OR = 11.15; 95% CI: 53.84-79.61, p < 0.0001). Other factors including the percentage of male respondents (80%; OR: 0.16-6.25; 95% CI: 0.593-0.932, p = 1) and the percentage of married persons (69%; OR: 0.35; 95% CI: 0.386-0.909, p = 0.221) did not show any significant connection. A significant link has been seen with patients under 30 years of age (50%; OR: 0.16; 95% CI: 0.118-0.882, p = 0.043). Furthermore, among those with any co-infection, there was a significant association with a p-value of 0.043 (Table 3).

Table 3 Demographic details of HIV-1 infected studied subjects

Variables	No. of collected Samples	No. of Positive samples	% HIV-1 Positive	95% Confidence Interval (C.I)	Odd Ratio (OR)	*p*-value
Gender						
Male	25	20	80	0.593-0.932	1 (0.16-6.25)	1^{NS}
Female	10	8	80	0.444-0.975		
Marital Status						
Married	13	9	69	0.386-0.909	0.35 (0.06-1.93)	0.221^{NS}
Unmarried	22	19	86	0.651-0.971		
Age (years)						
< 30 years	6	3	50	0.118-0.882	0.16 (0.02-1.09)	0.043^{S}
> 30 years	29	25	86	0.683-0.961		
Districts						
Lahore	15	14	93	0.680-0.998	4.67 (0.48-45.04)	0.154^{NS}
Other Districts	20	15	75	0.509-0.913		
Co-Infection if present						
No Infection	6	3	50	0.118-0.882	0.16 (0.02-1.09)	0.043^{S}
Infection Present	29	25	86	0.683-0.961		

Significant value (p<=0.05), NS= Non-significant

Pol gene amplification

Following amplification, the resulting PCR products having amplicon size of 1084 bp were visualized using a Gel doc imaging system (Figure 1), which captured an agarose gel image displaying five distinct PCR amplicons alongside a 1 kb DNA ladder for size reference.

Figure 1: Amplicons of 1084bp under gel doc system

After optimization, reverse transcriptase polymerase chain reaction (RT-PCR) was recycled to effectively strengthen HIV-1 pol gene (1084 bp) at a strengthening temperature of 54–57°C. A UV illuminator was used to see the amplified PCR products. An agarose gel with six different PCR amplicons and a 1 kb ladder can be seen in Figure 2.

Figure 2: Desired amplicons (Pol gene) of 1084bp after RT-PCR

Sanger Sequencing

Following amplification of HIV-1 pol gene amplicons, PCR products were sequenced using Sanger sequencing. The sequencing data was analyzed using BioEdit to identify specific sequence details and variations within the HIV-1 pol gene. Figure 3 presents the sequencing electropherogram or chromatogram obtained from the Sanger sequencing analysis. The chromatogram highlights key sequence features, including nucleotide base calls and potential variations such as point mutations or insertions/deletions within HIV-1 pol gene.

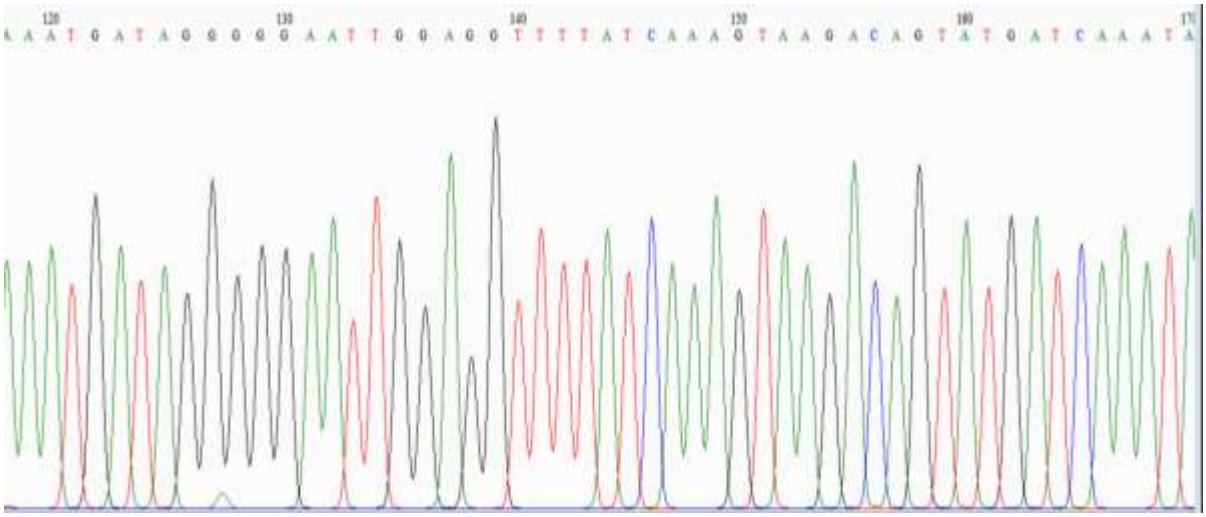

Figure 3: Electropherogram or Chromatogram Sequencing

HIV BLAST

The sequences obtained from the study were subjected to analysis using NCBI Basic Local Alignment Search Tool (BLAST) to evaluate their similarity to known sequences available in public databases. Figure 4 illustrates the consequences of the NCBI BLAST analysis, displaying the alignment and relatedness of the study's query sequence with other HIV-1 sequences retrieved from public databases.

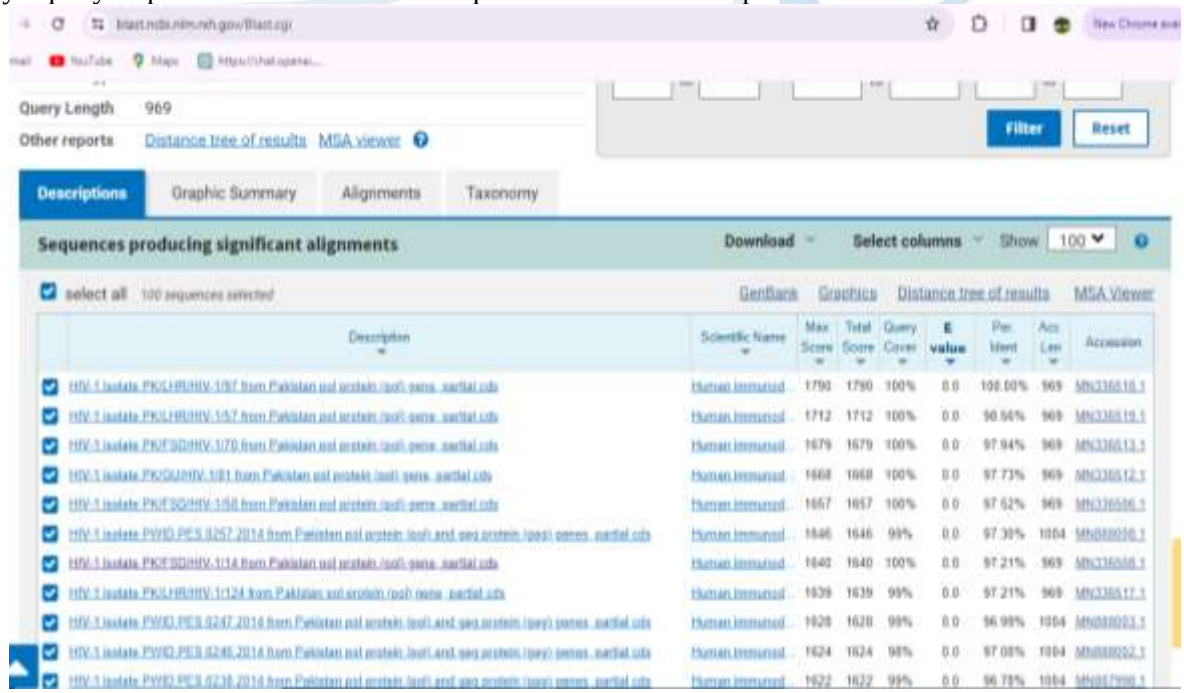

Figure 0: HIV BLAST result of query sequence available in NCBI HIV database

Genetic variety of HIV-1

The HIV-1 strains genetic diversity was examined by investigating pol gene sequences obtained from the study samples. Following a comprehensive examination and phylogenetic analysis, these sequences were categorized into distinct clades representing different HIV-1 subtypes. Fourteen (14) HIV-1 pol sequences corresponded to sub-subtype 02_AG, initiating from Ghana and Pakistan. This clustering indicates a prevalent presence of sub-subtype 02_AG within the study population, suggesting a genetic linkage between HIV strains in these regions. Two (2) sequences from Uganda, South Africa, and Pakistan formed a distinct clade indicative

of subtype A. This clustering highlights the genetic relatedness and geographic distribution of subtype A HIV-1 strains across multiple regions. Two (2) additional sequences from Kenya, Cameroon, and Ghana clustered within a clade representing subtype-G. Figure 5 visually represents the phylogenetic relationships among the analyzed HIV-1 pol gene sequences, highlighting the clustering patterns observed for subtypes 02_AG, A, and G.

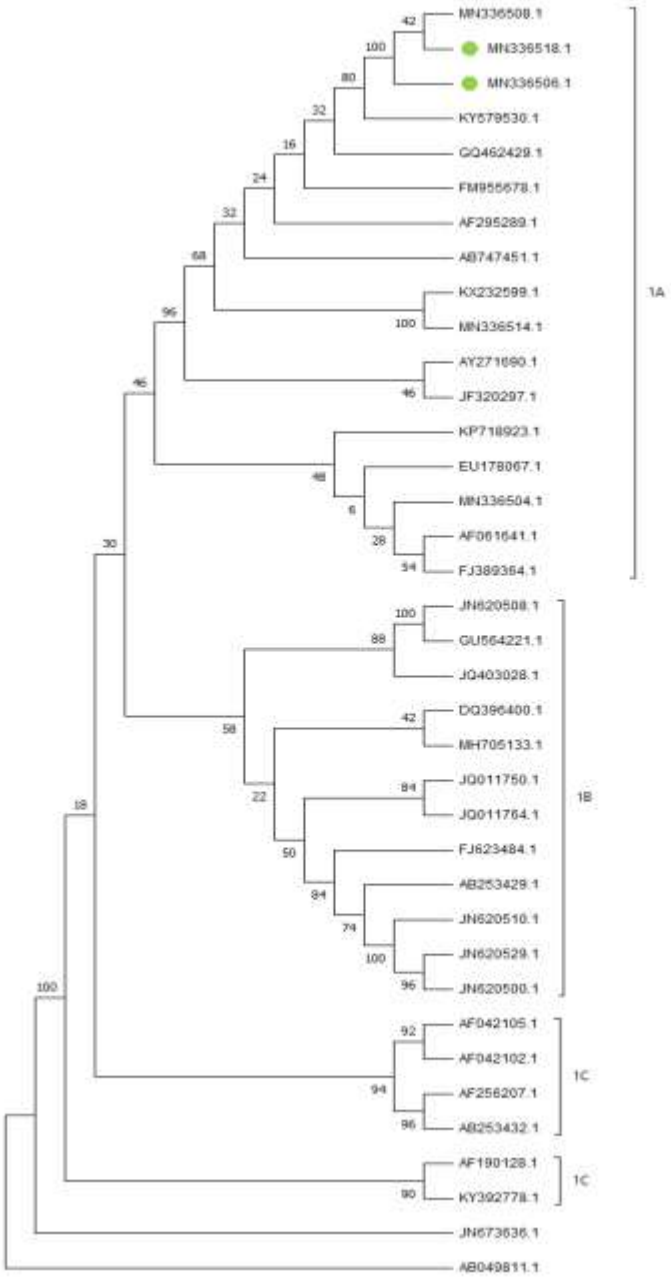

Figure 4: Phylogenetic tree of the pol gene (1084bp) using MEGA 7.0 software

Analysis of mutations and substitutions

Non-nucleoside-Analog-Reverse-Transcriptase-Inhibitors, Protease inhibitors, and Nucleoside-Analog-Reverse-Transcriptase-Inhibitors are anti-HIV medicines which are delivered in grouping at PACP

management centers [12]. Numerous substitutions were detected in study subjects at 113V, K14R, L19P, K20I, N37D, L63S, H69K, M36I, L89M through complete length of PR region.

Resistance mutations to nucleoside reverse transcriptase inhibitor (NRTI) at regions S68G, D67DN and V75VM were found. In NNRTI, resistance was found at E138A and V106I.

Other non-important substitutions that were detected through complete length of RT area were K32Q, V35T, E36I, K49R, I135V, D123E, S162A, T165I, Q174K, D177E, K173T, I178L, T200A, Q207E, P243A, V245Q, R356K, S322T, E291D, P294T, A272P, T286A, V292I, I293V, I326V, G335D, M357R, G359S, K366Q, T369A, E370G, A371V, I375V, T377I, K390S, K395R, A400T, T403M, E432D, R461K, D471E, K476R,Q480H, H483Y, K512R, L491S, L517I, S519N, Q524K, K527E, A534S , A554N, E529D.

In INSTI Accessory region of Integrase (IN), there was resistance mutation in E157Q only. No other mutation was observed in whole length of study sequence of IN region K14R, K136T, T112V, V31I, T124A, T125A, L101I, G134N, I135V, K160Q, V201I, I203M, T206S, I208L, T218I, L234I, A265V, S283G, D288N.

Analysis of drug resistance

Assessment of drug resistance in the PR region (1-90 codons), RT region (1-500 codons) and IN region (1–288 codons) revealed S68G, D67DN and V75VM mutations in the NRTI region. A mutation of E138A and V106I was reveled in NNRTI region. The consensus amino acid in CRF02-AG and subtype-G is K20I. K20I is a PI-selected mutation with unknown impact on currently utilized PIs in the PR region in subtypes C and B. An additional change M46K (indicated in red) was detected in the PR area of HIV-1 pol gene. In case of Integrase region, there was only one E157Q mutation in INSTI Accessory region (Figure 6).

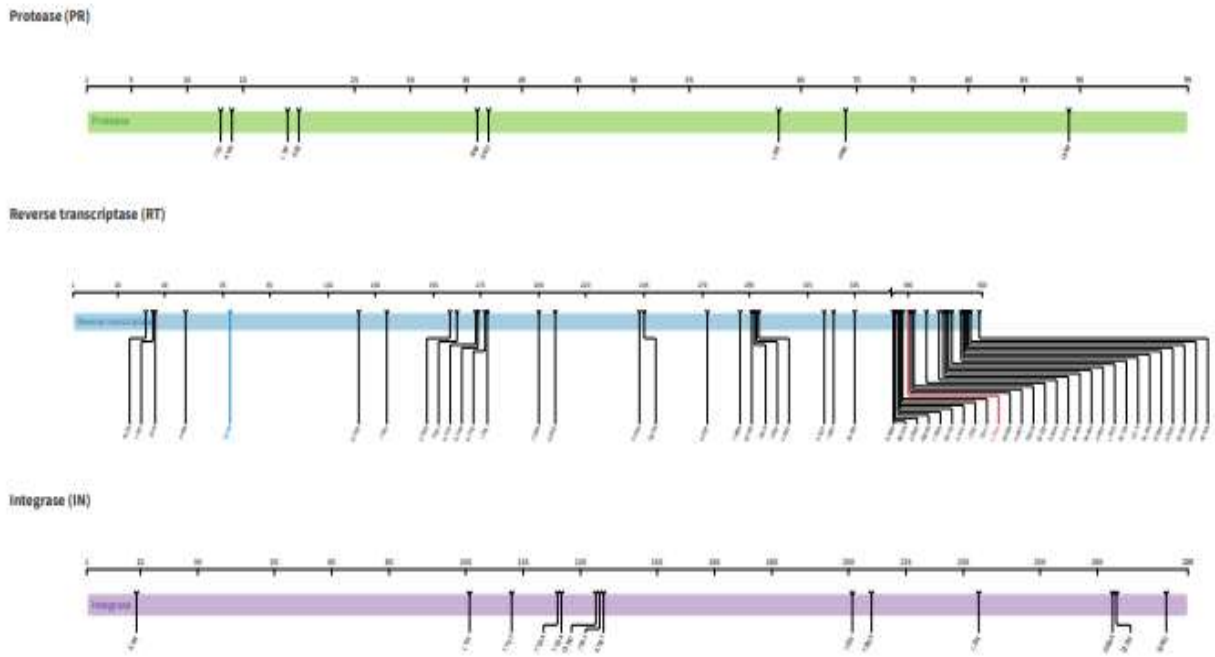

Figure 5: Drug resistance investigation on study isolate PK/LHR/HIV-1/90, 1/500, 1/288 using the Stanford HIV db Programme

Discussion

In our study, we studied PR, RT and IN gene-coding areas in 35 plasma samples. Initial screening test results indicated that 28 samples tested positive for HIV-1 (80%; 95% CI: 0.593-0.932). The screening test outcomes were in clear correlation with the RT-PCR findings, with all 35 samples testing positive for HIV-1 showing high accuracy of Alere HIV Combo test. The upper most occurrences were detected in different persons from Lahore, Gujranwala, Sargodha and Faisalabad. Individuals those having history of use of drug injection had

significantly higher likelihood of HIV-1 occurrence compared to others. No significant associations were found with other variables like gender or marital status. There was an association between individuals under 30 years old and those with any co-infection, indicating a higher risk of HIV-1 in these demographic categories. These results are in line with earlier research indicating high prevalence in Pakistan, emphasizing the urgent need for more comprehensive health education and targeted awareness programs, particularly in the Punjab province, to promote safe injection practices to avoid the spread of HIV-1 [17].

Drug injection use is a main threat issue that may expedite the development of HIV-1 infection, which is particularly concerning among transgender individuals in nations with low HIV incidence like Pakistan. Diagnostic centers that serve people involved in high-threat action similar to drug injection have a substantial function in blowout of HIV-1. The initial HIV epidemic amongst persons who inoculate drugs in Larkana highlighted entrenched nature of HIV-1 among drug abusers throughout the country [18]. Despite efforts to implement preventative measures, HIV epidemics continue to grow among drug inoculators over time. Furthermore, a significant association was seen in these individuals regarding the co-occurrence of HIV-1 and other coinfections. These findings are in line with other studies indicating co-infection with Hepatitis-C-Virus in HIV infected individuals [19].

We successfully amplified the HIV-1 pol-gene (1084 bp) using RT-PCR. For strain classification, we sequenced both the PR gene (30–297 bp) and the RT gene (123–720 bp) of HIV-1, which is required for minimal genotyping. The BLAST analysis revealed a 100% match between the query sequence and HIV-1 isolates from Pakistan, indicating a high degree of genetic similarity. This result recommends that the same HIV-1 strain is circulating in Pakistan. The 100% similarity is likely because of the minor sample size and the secrecy maintained during data collection, which limited the availability of diverse sequences for comparison. Nevertheless, this result highlights that viral RNA has not gain deadly mutations as of yet and current regimen of treatment can still be used effectively.

After conducting a meticulous analysis, the pol gene sequences under investigation were categorized into three distinct clades based on their phylogenetic relationships. Direct ancestral relationship is difficult to establish without knowledge about the patient's travel history. However, the grouping of subtypes 02_AG, G, and A in African nations implies that HIV-1 most likely started in Africa. The previously published data highlights the continuous fluctuation in the genetic composition and possible evolutionary patterns of HIV-1. Inter-subtype recombinant and multiple subtypes variants have resulted in succeeding generations at level of community [20].

Our analysis identified a predominant clustering of sequences with sub-subtype 02_AG, originating from Ghana and Pakistan. This clustering suggests that international migration or transmission networks involving individuals from Ghana are responsible for the viral transmission. The observation of sequences from Uganda, South Africa, and Pakistan clustering together in a subtype-A clade further underscores the international diversity of HIV-1 and highlights interconnectedness of HIV dispersion across geographical boundaries. Comparisons with sequences from Kenya, Cameroon, and Ghana clustering within a subtype G clade emphasize the complex genetic landscape of HIV-1, demonstrating co-circulation of multiple subtypes and recombinant forms within the study area. These findings align with previous studies documenting the diverse genetic makeup of HIV-1 in various regions [21].

Anti-HIV drugs inhibiting the enzymes coded by *pol* gene are available at PACP treatment centers free of charge. Thus, it was crucial to estimate sequences of the *pol* gene to explain substitutions linked with the drug resistance between mingling HIV-1 subtypes. From numerous substitutions which were detected in study subjects, a resistance mutation to NRTI (S68G, D67DN, V75VM) and to NNRTI at E138A and V106I were of significance in the overall length of PR-region. While, in INSTI Accessory region of Integrase (IN), there was resistance mutation in E157Q only. The reported findings align with an earlier study, where no significant resistance-associated-substitutions were detected in the study sequences [11]. When considered collectively, the analysis revealed a low existence of drug-resistance-associated mutations in the infected people. In light of this, these patients may eventually be treated with protease inhibitors in addition to reverse transcriptase inhibitors.

Information regarding drug-resistance-associated mutations and circulating subtypes of HIV-1 will be beneficial in helping the regulatory bodies to select the appropriate interventions and treatment plans. However, due to the limitation of small sample size and consistent findings, future large-scale studies are required to confirm these results. It is crucial that continuing drug resistance monitoring be implemented in order to choose the best possible treatment choices in our province.

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None

Conflict of Interest
None

Correspondence

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