

Emergence of Novel Unique Recombinant Forms and Multiple Subtypes in Gag-Pol Region of HIV-1 in Punjab, Pakistan

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ABSTRACT

The current era faces a major health challenge of drug resistance and viral escape mutants of human immunodeficiency virus (HIV-1) worldwide. Therefore, a deep molecular insight unveiling the genetic characteristics of the prevailing strains is the need of the hour for successful disease control interventions in endemic countries like Pakistan. Here we present, a detailed molecular and computational analysis of the major genetic constituents, Gag-Pol region of HIV-1 for the very first time in Punjab-Pakistan including three regions of outbreak i.e. Kot Imrana Sargodha, Faisalabad and Gujrat. Drug resistance mutations such as T12A, I13A, K14R, I15V, K20I, T31S, E35D, M36I, N37D, R41K, K43X, R57K, Q61H, H69K, K70R and L89M were observed across several sites across the whole length of PR region among the isolates. A significant accessory resistance mutation V32I conferring a low level of resistance against non-nucleoside reverse transcriptase inhibitors was observed among two isolates. This kind of genotypic pattern demonstrated a striking evidence of multiple transmission routes and co-circulating viruses due to increased genetic diversity in Pakistani isolates. However, SNAP analyses demonstrated higher values for Subtype A1 and Subtype C denoting a positive selection pressure and sequence conservation among the HIV-1 isolates in Pakistan. Our phylogenetic analyses revealed that HIV subtype A1 was found to be the most predominant subtype found in Pakistani population with 30% ratio among the isolates, 10% was of CRF02_AG, Subtype C a potential recombinant had 3.33% and unique recombinant form URFs accounted for 13.3% in the HIV Gag sequences. All Pol (URFs) demonstrated 40% of ratio among the isolates, and Subtype D with 6.66%. The present study shows that multiple subtypes of HIV-1 are present in the affected population. Hence, this study highlights the demand of continuous future studies on disease surveillance coupled with responsible evaluation of drug resistance in HIV-1 at higher resolution. This study emphasizes on the sudden emergence of circulating HIV-1 subtypes and its deadly drug resistant variants in Pakistan in the recent years. However, such molecular approaches in developing nations like Pakistan, will pave favourable strategies for advanced drug design, diagnostic assays and antiretroviral regimen therapies.

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AZ conceived, designed and wrote the research article. MAS proofread the article. HJ confirmed the data and sources. MAUK and ARS helped in writing of the article and analysis of data.

Key words

Gag-Pol, Unique recombinant forms (URFs), Protease inhibitors (PI), Mutations, Drug resistance

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV)

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which is a lentivirus and belongs to the family retroviridae. It is an RNA virus with a 9.2-kb genome that encodes structural and regulatory proteins in the order 5'-gag-pol-vif-vpr-tat-rev-vpuenv-3' (Girard *et al.*, 2006). HIV-1 probably originated from Africa from non-human primates and was later transferred to humans early in the 20th century (Worobey *et al.*, 2008). HIV-1 and HIV-2 are the two main types which initiate the onset of infection. HIV-1 having its dwelling roots from Southern Cameroon was transferred to humans from wild chimpanzees during the twentieth century (Gao *et al.*, 1999; Keele *et al.*, 2006). Its evolution was from a Simian immunode-

iciency virus. On the other hand, HIV-2 seems to have its roots in Sooty Mangabey (*Cercocebusatys*), Gabon, and Cameroon (Reeves and Doms, 2002). HIV-1 possesses a high genetic variation and the deficiency of proof reading in the viral transcriptase provokes an increased rate of recombination and genetic mutations frequently. These mutations have given rise to emerging escape mutants with the passage of time (Abram *et al.*, 2010). However, the increasing number of mutations in HIV-1 in the current situation has evolved to multiple genetic recombinants mainly such as circulating recombinant forms (CRFs) and unique recombinant forms (URFs) (Tongo *et al.*, 2016). The universal Los Alamos HIV database does however, displays many CRFs circulating around the world. Asian countries have a predominancy of Subtypes A, B, C, D and G with multiple CRFs (Junqueira *et al.*, 2016; Sanders-Buell *et al.*, 2010; Sarker *et al.*, 2008). In a developing country like Pakistan with a high burden of socio-economic conditions, HIV poses a significant health concern with the increasing number of cases and outbreaks. The trend has shifted from low to high rise in HIV infection in the country. Like any other developing country due to resource limited settings, AIDS is a major public health burden in Pakistan due to increase in HIV-1 positive cases in the near past. Recent reports of NACP ranked Punjab with the highest number of HIV cases of 75000, Sindh with 60,000, Balochistan as 5275 followed by Khyber Pakhtunkhwa with 16322 cases (Khanani *et al.*, 1988). Urban development, migration from smaller cities for education, employment and medical facilities contributed to population rise mainly in the Punjab region. Thus migrational influx however could be one of the possible reasons of the increased number of HIV-1 cases. In low income nations like Pakistan, monitoring of HIV drug resistance and population-based surveillances are highly the need of the hour, to ensure the efficacy of continuing the treatment regimen for HIV-1 (Bennett *et al.*, 2006; Jordan *et al.*, 2008; Gilks *et al.*, 2006). Increased urbanization in Punjab contributes as a major facilitating factor for exploration of HIV-1 subtypes, drug resistance mutations and genetic variations. Pakistan, however, demonstrates paucity in molecular and computational HIV related studies to date. This study focusses on investigating the drug-resistance mechanisms of the circulating HIV-1 subtypes in Punjab and highlighting the phylogenetic relationship among the isolates. The recombinant strains and URFs discussed in this study are however reported for the first time in both *Gag* and *Pol* genes in HIV-1 patients in Punjab, Pakistan. The data analyzed in the current study, unveils for the first time the major targeted areas of outbreak in Punjab for both *Gag* and *Pol* genes molecularly and computationally. Hence, such studies on this deadly virus will provoke necessary health implementations for

treatment and control of HIV-1 in Pakistan in the near future.

MATERIALS AND METHODS

HIV-1 positive samples were directed from different treatment centers, to Punjab AIDS Control Programme (PACP) Lahore between October 2018 to March 2020. Among them only 122 HIV-1 positive samples were selected with increased viral load for the current study. Only 30 HIV-1 (0.1%) positive isolates fulfilled the quality processing criteria for genotype analysis. Informed consent was taken from all the selected subjects. RNA was extracted from 200 µl of plasma by QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. cDNA was prepared by the Revert Aid First Strand method. Both *Gag* (HXB2:781–1861) and *Pol* fragments were amplified (HXB2: 2147–3462) in a nested polymerase chain reaction (Chen *et al.*, 2014). The successfully amplified samples for HIV-1 *Gag* and *Pol* were used for genotyping and phylogenetic analysis with their mentioned sociodemographics (Table I). Amplified samples were sequenced by First Base Laboratories Sdn Bhd, Seri Kembangan, and Selangor, Malaysia on an ABI 3730xl automated DNA analyzer (Applied Biosystems, Foster City, CA). Edited sequences were evaluated for drug resistance mutations using the surveillance drug resistance mutations (SDRM) software and the Stanford University Algorithm (<http://hivdb.stanford.edu>). REGA HIV subtyping tool was used for subtyping of *Gag* and *Pol* (<http://www.bioafrica.net/subtypetool/html/>). Intersubtype mosaicism in the potential sequences was investigated by the Recombination Identification Program (RIP version 3.5.1). The neighbor-joining method using MEGA (Molecular Evolutionary Genetics Analysis, version 5.1) was used for phylogenetic analyses (Tamura *et al.*, 2007). For non synonymous-synonymous substitution (dn-ds) analysis of sequences SNAP programme was used. DNA sequences were aligned using CLUSTAL W (Huang *et al.*, 2012; Wilkinson *et al.*, 2014). The HIV-1 isolates of *pol* gene, were analyzed for RT and PR regions by Calibrated Population Resistance (CPR) Tool (Version 6.0) (Chen *et al.*, 2012). HIV-1 sequences were then submitted to GenBank under the following accession numbers: MT462197-MT462205, MT436255-MT436257, MT489302-MT489309 and MT561289- MT561297. The *Gag* and *Pol* sequences were edited using Bio-Edit 7.0 software.

RESULTS

Demographic characteristics of study isolates

The isolates selected in the current study were located in the districts of Lahore (n =54), Faisalabad (n= 38),

Jalalpur Jattan-Gujrat (n= 32), Kot Imrana-Sargodha (n= 42), Sheikhpura (n=13), Kasur (n=21), Multan (n =29), Bhawalpur (n=18), Rawalpindi (n=31), Jhelum (n =13), Layyah (n=17) and Rahim Yar Khan (n =11) in Punjab. [Table I](#) shows the demographic characteristics of the 30 selected subjects in the Punjab for this study. The ratio of males to females was 63.3% in males and 26.6% in females

among the 30 isolates with mean age as ± 32 years. Lahore being the major suburb had a ratio of 16.6% followed by 10% for Sargodha, 6.66% Multan, 6.66% Rawalpindi, 10% Bhawalpur, 6.66% Sheikhpura, 3.33% Jhelum, 6.66% Rahim Yar Khan, 10% Faisalabad, 10% Layyah, 6.66% Kasur and 13.3% in Gujrat. Among these regions we

Table I. Socio-demographic features and genotypes of the study subjects.

Isolate ID	Age	Gender	Transmission route	City	Gene HIV subtype	CD4 count	Drug resistant mutations
MBBL01	26	Male	IDU	Lahore	<i>Gag</i> Subtype A1	383	
MBBL02	31	Male	IDU	Lahore	<i>Gag</i> Subtype A1	506	
MBBL03	44	Male	IDU	Lahore	<i>Gag</i> Subtype A1	149	
MBBL04	29	Male	IDU	Sargodha	<i>Gag</i> CRF02_AG	225	
MBBL05	55	Male	Immigrant worker	Multan	<i>Gag</i> CRF02_AG	280	
MBBL06	47	Male	Quack treatment	Rawalpindi	<i>Gag</i> Subtype A1	422	
MBBL07	42	Female	Quack treatment	Bhawalpur	<i>Gag</i> Subtype A1	137	
MBBL08	36	Female	Surgeries	Sheikhpura	<i>Gag</i> Subtype C/ Potential recombinant	335	
MBBL09	33	Female	FSW	Rawalpindi	<i>Gag</i> Subtype A1	196	
MBBL10	29	Transgender	Sex	Multan	<i>Gag</i> CRF02_AG	882	
MBBL11	41	Transgender	Sex	Jhelum	<i>Pol</i> URF/A1+G	341	
MBBL12	50	Male	Immigrant worker	Rawalpindi	<i>Gag</i> Subtype A1/URF	400	
MBBL13	38	Male	IDU	Kot Momin-Sargodha	<i>Gag</i> Subtype A1	475	
MBBL14	26	Male	IDU	Kot Momin- Sargodha	<i>Gag</i> Subtype A1	687	
MBBL15	37	Female	Spouse HIV positive	Sargodha	<i>Pol</i> URF	474	NRTI/PI
MBBL16	32	Male	IDU	Rahim Yar Khan	<i>Pol</i> URF	124	
MBBL17	44	Male	IDU	Lahore	<i>Pol</i> URF	225	PI/PI SDRM
MBBL18	23	Female	FSW	Multan	<i>Pol</i> URF	109	NRTI
MBBL19	33	Male	Immigrant worker	Bhawalpur	<i>Pol</i> URF	256	NRTI
MBBL20	27	Female	FSW	Faisalabad	<i>Pol</i> URF	338	NNRTI
MBBL21	22	Male	Quack treatment	Faisalabad	<i>Pol</i> URF	117	NRTI/PI
MBBL22	34	Male	IDU	Jalalpur Jattan-Gujrat	<i>Pol</i> URF	90	
MBBL23	25	Male	IDU	Gujrat	<i>Pol</i> URF	678	NRTI/PI
MBBL24	28	Male	MSM	Jhelum	<i>Pol</i> URF	567	NRTI/NNRTI
MBBL25	31	Male	MSM	Layyah	<i>Pol</i> URF	347	NNRTI
MBBL26	22	Female	Spouse HIV positive	Jalalpur Jattan-Gujrat	<i>Pol</i> Subtype A1/ URF	109	NNRTI
MBBL27	30	Male	Immigrant worker	Faisalabad	<i>Gag</i> CRF02_AG	415	
MBBL28	25	Transgender	Sex	Lahore	<i>Gag</i> Subtype A1	223	
MBBL29	29	Male	IDU	Kasur	<i>Gag</i> Subtype A1	446	
MBBL30	39	Female	Spouse HIV positive	Kot Momin -Sargodha	<i>Gag</i> Subtype A1/ URF	258	NA

For abbreviation see [Table I](#).

present for the very first time molecular and computational analysis of Gag-Pol from Punjab region including three regions of outbreak Kot Imrana Sargodha, Faisalabad and Gujrat. The major transmission routes among the detected subjects were heterosexual transmission accounting of 16.6% as Immigrant workers, 10% as FSW, 6.66% as infection through spouse and 6.66% as transgenders. Intravenous drug transmission ranked the second highest route with 26.6% while the other transmission routes i.e. 10% as homosexual transmission and unknown routes accounted for 6.66% of infections (Table I).

HIV genotyping analysis

Prediction of genetic variability reveals all the possible viral evolutionary information. Therefore, HIV-1 *Gag* and *Pol* sequences were analyzed by REGA HIV subtyping tool for subtype determination (Jeannot *et al.*, 2009). HIV subtype A1 was found to be the most predominant subtype found in Pakistani population with 30% ratio among the isolates. CRF02_AG accounted for 10%, Subtype C a potential recombinant had 3.33% and the Unique Recombinant forms (URFs) accounted for 13.3% in the HIV *Gag* sequences (Table I). Boot scanning analysis of the *Gag* sequences however revealed four unique recombinant forms as shown in Table I. All the isolate sequences were subjected with bootscan cluster support 1.0 and 0.87 with a confidence level of 99.9%. The prevalence of URFs 13.3 % and CRF02_AG 10% was synchronous with each other which strongly strikes towards the notion that there might have been frequent recombination among the subtype A1 main strains itself (Table I). The subtype A1 in Pakistani population was significant among the IDUs. Isolate MBBL03, MBBL09, MBBL12 and MBBL30, demonstrated isolate MBBL03, MBBL09, MBBL12 and MBBL30, demonstrated HIV Subtype A1 as shown in Figure 1. Among the *pol* sequences, bootscanning analysis revealed that maximum number of subtype determination was accounted for unique recombinant forms (URFs) among the 13 isolates (Table II, Fig. 3). Intersubtype recombination was demonstrated strongly among the *pol* genotyped isolates. All *Pol* URFs demonstrated 40% of ratio among the isolates, and subtype D with 6.66% (Table II). The subtype genetic variability and diversity varied with different routes of transmission in infection (Table II). HIV infection caused due to quack treatment also demonstrated subtype A1 prevalence as it is caused by common syringe usage. Some IDUs and 2 isolates MBBL06 and MBBL07 were quack infected and demonstrated genotype A1 (Table I). However, two isolates MBBL05 and MBBL27 did show the genotype CRF02_AG which does suggest the notion that there might be a strong heterosexual transmission route from

expats returning from abroad depicting this genotype (Fig. 2A-C). This kind of genotypic pattern shows an association with multiple transmission routes (Tables I and II). The proportion of URFs among the *pol* genotyped isolates was significantly higher than *Gag*. This increased prevalence strikes towards an increased vulnerable hypervirulence in the HIV virus (Zahra *et al.*, 2021).

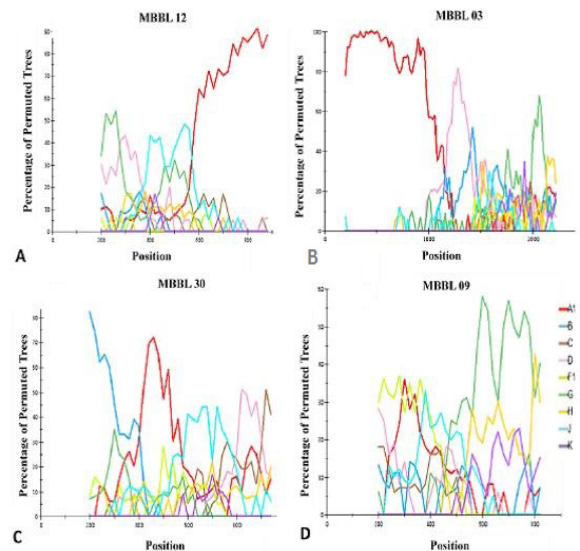


Fig. 1. Genotypic analysis of HIV-1 *Gag* sequences: Boot scanning analysis of Subtype A1 in HIV-1 *Gag* gene region. (A) isolate MBBL12 (B) isolate MBBL03 (C) isolate MBBL30 (D) isolate MBBL09. Parametrical analysis was done using the following: Window: 200 bp, step: 20 bp, gapstrip: on, reps: 100, Kimura (2-parameter), T/t: 2.0.

Recombination and substitution analysis

Genetic diversity initiates intersubtype mosaicism among the candidate genes. There was a high rate of recombination among the isolates identified with *Gag* gene in our study (Supplementary Fig. 1). Window size= 200, Confidence threshold = 0.9, Gap option= 3, and Multistate characters = yes, were used for the recombination analysis of the isolates. The RIP analysis of these four isolates MBBL01, MBBL05, MBBL14 and MBBL10 demonstrated a similarity score of 0.87 (Supplementary Fig. 1A), 0.91 (Supplementary Fig. 1B), 0.92 (Supplementary Fig. 1C), and 0.89 (Supplementary Fig. 1D). This increased level of mosaicism however denotes to a high rate of intersubtype mosaicism occurring among the region. However previous studies in Pakistan did demonstrate this fact about co-circulating viruses due to increased genetic diversity (Abidi *et al.*, 2014). To measure the rate of natural selection, 30 isolate sequences

were subjected to SNAP programme for computing dn/ds ratio. The SNAP results however revealed higher values for Subtype A1 and Subtype C denoting a positive selection pressure and sequence conservation within the Pakistani population. Cumulative behavior by SNAP analysis was shown in [Supplementary Figure S2](#) and [Supplementary Table SI](#).

Genotypic analysis of drug resistance

Anti HIV drugs such as nucleoside reverse transcriptase inhibitors, protease inhibitors and non-nucleoside analog reverse transcriptase inhibitors are generally the recommended combination of drugs used by HIV-1 infected individuals in Punjab, Pakistan as provided at PACP treatment centers. Substitution mutations with a high degree of drug resistance poses a challenge on the treatment and efficacy of HIV-1. Hence, to combat the hinderances evaluation of the RT and PR region in *Pol*

gene would pave pathways in exploring the associated drug resistance in circulating HIV-1 subtypes. Our analysis demonstrated an accessory resistance mutation V32I only in one isolate MBBL17 originating from Lahore. These findings however exhibit a synchronization with previous studies done in Sindh-Pakistan where a single primary mutation (L90M) was detected in a patient with PI usage history ([Shah et al., 2011](#)). The drug resistance analysis in our study neither showed major nor minor mutations significantly suggesting the fact that HIV-1 strains are sensitive to NNRTIS and NRTIS being used in Pakistan. Our investigations also shed light on the notion that the circulating viruses reflect sensitivity especially towards protease inhibitors like lopinavir, darunavir and atazanavir. Some non-significant substitutions were observed in the whole length of the RT region. The substitutions were: V35T, D121Y, V60I, K49R, I135T, K122E, I142L, K166R, Q207G, R211K, A272P, K275R, K277R, Q278H,

Table II. Demographic and genetic characteristics of HIV-1 isolates with URFs in the *Pol* gene.

Isolate ID	Gender	Age	Route of transmission	City	CD4 count	Gene	Sub-type	Drug resistant mutations			
								NRTI	NNRTI	PI	PI SDRM
MBBL15	Female	37	Spouse HIV positive	Sargodha	474	Pol	URF	Q58P, Q61X		M66I, 147 V, D60G, 189 M	
MBBL17	Male	44	IDU	Lahore	225	Pol	URF			E21X, D29X, D3C	V32I
MBBL18	Female	23	FSW	Multan	109	Pol	URF	K43X			
MBBL19	Male	33	Immigrant Worker	Bhawalpur	256	Pol	URF	K43X			
MBBL20	Female	27	FSW	Faisalabad	338	Pol	URF		Y188NY, H198HP, W229RW		
MBBL21	Male	22	Quack Treatment	Faisalabad	117	Pol	URF	R78RT, R172E, D1DE		L5V, R8L, P9A, A62AG, N54NY, D186DE	
MBBL23	Male	25	IDU	Gujrat	678	Pol	URF	R78RT, R172E, D1DE		L5V, R8L, P9A, A62G, I167N	
MBBL24	Male	28	MSM	Jhelum	567	Pol	URF	Q222R, E194X, L234P	Y181D, Y188N, E194X, H198L, K220R, G231R, W229X, Y232N, W239C		
MBBL25	Male	31	MSM	Layyah	347	Pol	URF		Y188N, H198X, K201Q, P217X, W229		
MBBL26	Female	22	Spouse HIV positive	Jalapur Jattan-Gujrat	109	Pol	Subtype D/URF		Y188NY, H198HP, W229RW		

Table Legend: IDU, intravenous drug users; FSW, female sex workers; MSM, males who have sex with males; URFs, Unique recombinant forms.

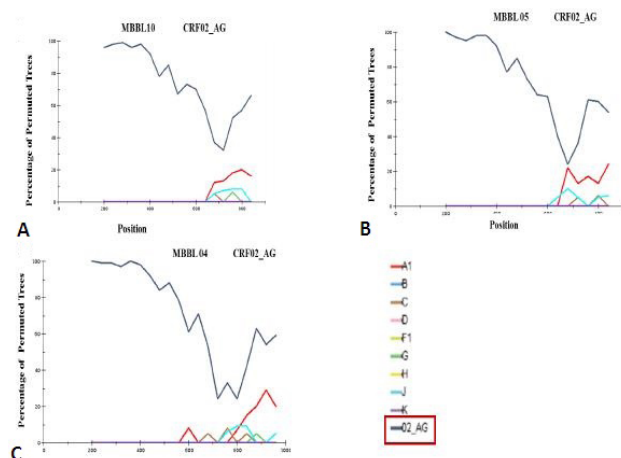


Fig. 2. Genotypic analysis of HIV-1 Gag circulating recombinant forms (CRFs): Boot scanning analysis of HIV-1 *Gag* gene with CRFs. (A) isolate MBBL10; (B) isolate MBBL05; (C) isolate MBBL 04. Conditions used were the following: Window: 400 bp, step size: 40 bp, gapstrip: on, reps: 100, Kimura (2-parameter), T/t: 2.0.

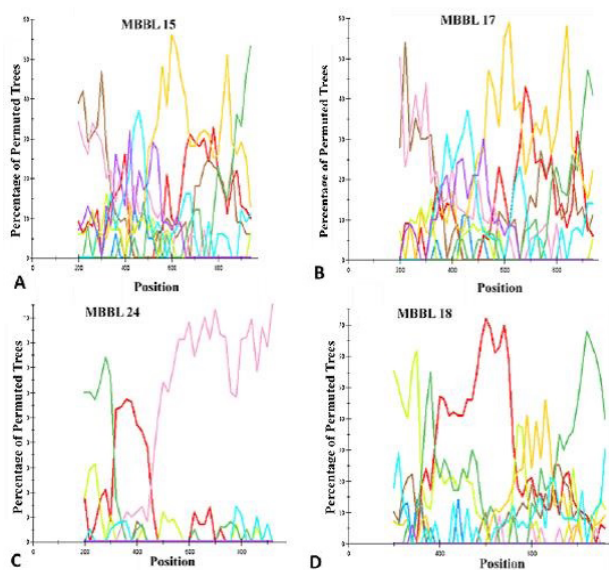


Fig. 3. Genotypic analysis of Pol Unique Recombinant Forms (URFs): Boot scanning analysis of HIV-1 *Pol* gene with URFs. (A) isolate MBBL15; (B) isolate MBBL17; (C) isolate MBBL 24; (D) isolate MBBL18. The conditions used for this analysis were the following: Window: 400 bp, step size: 40 bp, gapstrip: on, reps: 100, Kimura (2-parameter), T/t.

L282C, L283I and R248X in the isolates MBBL15, MBBL16, MBBL17, MBBL18, MBBL19 and MBBL20. The RT region exhibited minor mutations leaving an insignificant impact on drug resistance to date (Rhee *et*

al., 2003; Lai *et al.*, 2014; Gatanaga *et al.*, 2010). The current combination of drugs being used for HIV-1 so far in Pakistan showed low prevalence for drug resistance thus satisfying the continuation of these drugs for HIV-1 patients in Pakistan. However, non-significant substitutions such as T12A, I13A, K14R, I15V, K20I, T31S, E35D, M36I, N37D, R41K, K43X, R57K, Q61H, H69K, K70R and L89M were observed in the study subjects (MBBL18, MBBL19, MBBL20, MBBL21, MBBL23, MBBL24, MBBL25 and MBBL26) at several sites across the whole length of PR region. Q58P, Q61X, K43X, R78RT, R172E, D186DE, Q222R, E194X and L234P were identified as unique NRTI mutations. Among the NNRTI mutations Y188NY, H198HP, W229RW, Y181D, Y188N, E194X, H198L, K220R, G231R, W229X, Y232N, W239C, Y188N, H198X, K201Q, P217X, W229, Y1888NY, H198HP and W229RW were identified among the isolates (Table II). M66I, I47V, D60G, I89M, E21X, D29X, D30C, L5V, R8L, P9A, A62AG, N54NY, D186DE, L5V, R8L, P9A and I167N were the detected PIs. Only one isolate MBBL17 from Lahore had an accessory mutation V32I. Similarly, absence of substantial changes in the RT region denoted a conferred resistance to reverse transcriptase inhibitors (RTIs). Only one isolate had a mutation V32I which conferred a low level resistance to NNRTIs (Table II). Medium and low resistance levels to six PIs were detected against atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), saquinavir (SQV) and tipranavir (TPV). High-level and moderate resistances were also revealed among six NRTIs such as lamivudine (3TC), zidovudine (AZT), abacavir (ABC), stavudine (D4T), didanosine (DDI) and entricitabine (FTC) and four NNRTIs [efavirenz (EFV), etravirine (ETR), nevirapine (NVP) and rilpivirine (RPV)].

Phylogenetic analysis

Phylogenetic analysis demonstrated that 17 Gag sequences from this study clustered into three different clades. Among the isolates, 10 clustered with subtype A originating from Pakistan, Kenya, Uganda and Rwanda. Of these, 3 clustered with sequences corresponding sub-subtype 02_AG, having its descent from Kenya, Ghana and South Africa. Three isolates demonstrated URFs and 1 sequence clustered within a clade representing subtype A and G clustered within a clade representing sub-type G from Kenya, Cameroon and Ghana (Fig. 4A, B), showing an evolutionary origin from African countries. The isolate's travel histories were insufficient to have reasonably connected probable ancestral links. As a result, clustering of subtypes 02 AG, A, and G in African countries, therefore brought to evidence an evolutionary origin of HIV-1 from Africa (Huet *et al.*, 1990). However, 13 Pol

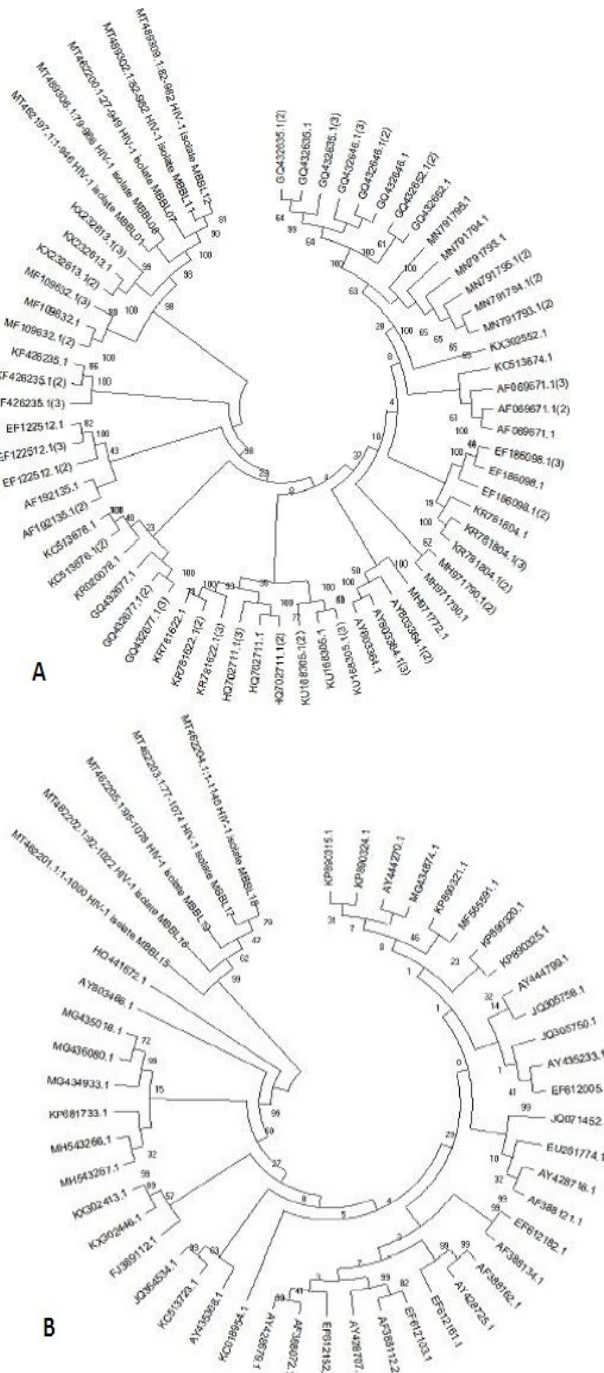


Fig. 4. Phylogenetic tree of 17 *Gag* isolates (A) and 13 *pol* isolates (B), generated via Neighbor Joining method. The distances were computed using the Kimura 2- parameter model. Evolutionary analysis was conducted in MEGA X. Sum of branch optimum length = 0.66910448. 1000 bootstrap test replicates are shown near to scale with branch lengths as those of the evolutionary distances used to infer the phylogenetic tree.

sequences were identified in our study, among them the highest ratio was of URFs. Forty percent of the sequences clustered with unique recombinant forms and only one isolate MBBL26 clustered with subtype D. This nature of clustering isolates with a wide range of reported databases therefore, indicates the genetic diversity, heterogeneity and potential evolutionary dynamics of HIV-1. These analyses give the notion of presence of many subtypes and inter-subtype recombinant forms at the population level (Song *et al.*, 2018).

DISCUSSION

Investigations based on HIV-1 *Gag* and *Pol* analysis among the sequences targeting the circulating genotypes in the Punjab Province was the main aim of the current study. The sociodemographic analysis of this study however, revealed that Lahore due to its urbanization and economic development does have the highest number of HIV-1 patients in the Punjab including three major regions of outbreak i.e., Kot Imrana (Sargodha), Gujrat (Jalalpur Jattan) and Faisalabad. In the regions of outbreak common syringe usage by the local quacks, and rural medical facilities was the main culprit of the outbreak (Zaid and Afzal 2018; Wahid, 2019). These findings do support the fact that indirect facilitation and fast population mobility could possibly be the major routes in spreading of HIV-1 and transmitting the viruses to the community as well. In this study, HIV-1 genotypes prevailing predominantly in the heterosexually infected population in Punjab were investigated based on the analysis of HIV-1 *Gag* and *Pol* sequences. Lahore being the capital of Punjab province could possibly lead to increased genetic diversity of HIV-1 due to its growing population. Lack of proper biosafety measures in hospitals, frequent migration in the suburb from nearby regions for education and health has contributed to the rise in infection (Rana and Bhatti, 2018). To add to the fact, previously reported researches however have brought to light the phenomenon of HIV-1 infection among people migrating frequently resulting in emergence of novel subtypes, inter-subtype recombinants, and CRFs (Zhong *et al.*, 2007; Kalichman *et al.*, 2017; Lebedev *et al.*, 2019). The subtype, SNAP and RIP analysis of this study however reveals major molecular revelations in HIV-1 sequences from Punjab, Pakistan. Nonetheless, phylogenetic and genotypic analyses of drug resistance in HIV-1 sequences brings to light the major significant and non-significant mutations especially in the *Pol* region. Such novel molecular analysis from Punjab, Pakistan, reported in our study could possibly pave pathways in understanding of the underlying mechanisms for HIV-1 in resource limited countries like Pakistan. HIV-1 subtype

distribution appears to be quite diverse in other Asian countries too. In Iran previous studies have reported HIV-1 subtypes A and B mainly in the IDUs (Sarrami-Forooshani *et al.*, 2006). Similarly, studies reported from countries like Uzbekistan, Kazakhstan and Yemen also demonstrated a prevalence of subtypes A, B and CRF02_AG which share synchrony with the present data indicating the commonly circulating prevalent subtypes in Asia (Kurbanov *et al.*, 2003; Carr *et al.*, 2005; Bobkov *et al.*, 2004; Saad *et al.*, 2005). HIV molecular epidemiological research in Pakistan is still in the infant stages, and yet well affirmed HIV prevention policies need to be designed. Previously reported data in Pakistan in HIV-1 genotypes evidently demonstrates genetic diversity with existing co-circulating genotypes such as: CRF_AG, A, B and CRF_AE which are synchronous with our findings also (Mujeeb and Altaf, 2003; Altaf and Mujeeb, 2002; Hyder and Khan, 1998). However, our data demonstrates that apart from Subtype A1, the URFs were becoming more prevalent than the previously reported subtypes A, G and CRF_02 AG (Khan *et al.*, 2006; Yaqub *et al.*, 2019). This changing trend of introduction of novel and new recombinant subtypes is because of returning migrant workers and expatriates in Pakistan. This unchecked migrational influx of expatriates, ultimately provokes increased incidence of HIV-1 infection. It has also contributed in altering the pattern of different HIV-1 subtypes and increased drug resistances turning it to a more challengeable situation for the concerned health authorities. Absence of major resistance-associated substitutions in our findings likewise agree with another study conducted in Sindh, Pakistan, where no major resistances were recognized (Baqi *et al.*, 1998; Khanani *et al.*, 2011; Khan *et al.*, 2018). Our study does come up with the notion that circulating viruses are highly sensitive to all protease inhibitors, implicating lopinavir, atazanavir and darunavir. The 30 isolates in this investigation had no significant major or minor mutations in the RT area that conferred resistance to reverse transcriptase inhibitors (RTIs). This strongly denotes the fact, that the prevalent HIV-1 strains in Pakistan are sensitive to the NRTIs and NNRTIs. PR and RT regions display down regulation in prevalence of drug-resistance associated mutations in the infected individuals. Combination drug regimens of PIs in combination with RTIs like lopinavir, ritonavir, nevirapine, lamivudine, tenofovir and zidovudine, could be continued to treat patients. Furthermore, continuation of these studies pave ways for essential modifications in drug regimens which could further be applicable for effective therapeutics and subsequent control in the future. Hence, the current study unveils novel information on circulating subtypes and drug resistance mutations in *Gag-Pol* genes for HIV-1 patients for the first time in Pakistan's

Punjab province. The phylogenetic and computational revelations made by this study will be extremely beneficial for clinicians, physicians and researchers in determining the best possible therapeutic strategies for controlling the transmission of this virus. It also uncovers primitive information about the evolutionary links and drug resistance patterns of the recent existing HIV strains in a developing country like Pakistan. Future studies are therefore highly the need of the hour to control the spread of this virus and monitor drug resistance mechanisms for implementation of appropriate therapeutic options.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20210824080807>

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

- Abidi, S.H., Kalish, M.L., Abbas, F., Rowland-Jones, S. and Ali, S., 2014. HIV-1 subtype A gag variability and epitope evolution. *PLoS One*, **9**: e93415. <https://doi.org/10.1371/journal.pone.0093415>
- Abram, M.E., Ferris, A.L., Shao, W., Alvord, W.G. and Hughes, S.H., 2010. Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. *J. Virol.*, **84**: 9864-9878. <https://doi.org/10.1128/JVI.00915-10>
- Altaf, A. and Mujeeb, S.A., 2002. Unsafe disposal of medical waste: A threat to the community and environment. *J. Pak. med. Assoc.*, **56**: 232-233.
- Baqi, S., Nabi, N., Hasan, S.N., Khan, A.J., Pasha, O.,

- Kayani, N., Haque, R.A., Khurshid, M., Fisher-Hoch, S., Luby, S.P. and McCormick, J.B., 1998. HIV antibody seroprevalence and associated risk factors in sex workers, drug users, and prisoners in Sindh, Pakistan. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.*, **18**: 73-79. <https://doi.org/10.1097/00042560-199805010-00011>
- Bennett, D.E., 2006. The requirement for surveillance of HIV drug resistance within antiretroviral rollout in the developing world. *Curr. Opin. Infect. Dis.*, **19**: 607-614. <https://doi.org/10.1097/QCO.0b013e3280109ff1>
- Bobkov, A.F., Kazennova, E.V., Sukhanova, A.L., Bobkova, M.R., Pokrovsky, V.V., Zeman, V.V., Kovtunen, N.G. and Erasilova, I.B., 2004. An HIV type 1 subtype A outbreak among injecting drug users in Kazakhstan. *AIDS Res. Hum. Retrovir.*, **20**: 1134-1136. <https://doi.org/10.1089/aid.2004.20.1134>
- Carr, J.K., Nadai, Y., Eyzaguirre, L., Saad, M.D., Khakimov, M.M., Yakubov, S.K., Birx, D.L., Graham, R.R., Wolfe, N.D., Earhart, K.C. and Sanchez, J.L., 2005. Outbreak of a West African recombinant of HIV-1 in Tashkent, Uzbekistan. *J. Acquir. Immune Defic. Syndr.*, **39**: 570-575.
- Chen, M., Ma, Y., Duan, S., Xing, H., Yao, S., Su, Y., Luo, H., Yang, L., Chen, H., Fu, L. and Qu, A., 2012. Genetic diversity and drug resistance among newly diagnosed and antiretroviral treatment-naïve HIV-infected individuals in western Yunnan: A hot area of viral recombination in China. *BMC Infect. Dis.*, **12**: 382. <https://doi.org/10.1186/1471-2334-12-382>
- Chen, M., Ma, Y., Su, Y., Yang, L., Zhang, R., Yang, C., Chen, H., Yan, W., Shi, Y., Dong, L. and Chen, L., 2014. HIV-1 genetic characteristics and transmitted drug resistance among men who have sex with men in Kunming, China. *PLoS One*, **9**: e87033. <https://doi.org/10.1371/journal.pone.0087033>
- Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M. and Sharp, P.M., 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature*, **397**: 436-441. <https://doi.org/10.1038/17130>
- Gatanaga, H., Ode, H., Hachiya, A., Hayashida, T., Sato, H. and Oka, S., 2010. Combination of V106I and V179D polymorphic mutations in human immunodeficiency virus type 1 reverse transcriptase confers resistance to efavirenz and nevirapine but not etravirine. *Antimicrob. Agents Chemother.*, **54**: 1596-1602. <https://doi.org/10.1128/AAC.01480-09>
- Gilks, C.F., Crowley, S., Ekpini, R., Gove, S., Perriens, J., Souteyrand, Y., Sutherland, D., Vitoria, M., Guerna, T. and De Cock, K., 2006. The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. *Lancet*, **368**: 505-510. [https://doi.org/10.1016/S0140-6736\(06\)69158-7](https://doi.org/10.1016/S0140-6736(06)69158-7)
- Girard, M.P., Osmanov, S.K. and Kieny, M.P., 2006. A review of vaccine research and development: the human immunodeficiency virus (HIV). *Vaccine*, **24**: 4062-4081. <https://doi.org/10.1016/j.vaccine.2006.02.031>
- Huang, A., Hogan, J.W., Istrail, S., DeLong, A., Katzenstein, D.A. and Kantor, R., 2012. Global analysis of sequence diversity within HIV-1 subtypes across geographic regions. *Future Virol.*, **7**: 505-517. <https://doi.org/10.2217/fvl.12.37>
- Huet, T., Cheynier, R., Meyerhans, A., Roelants, G. and Wain-Hobson, S., 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature*, **345**: 356-359. <https://doi.org/10.1038/345356a0>
- Hyder, A.A. and Khan, O.A., 1998. HIV/AIDS in Pakistan: The context and magnitude of an emerging threat. 1998. *J. Epidemiol. Commun. Hlth.*, **52**: 579-585. <https://doi.org/10.1136/jech.52.9.579>
- Jeannot, A.C., Reigadas, S., Schrive, M.H., Pinson, P. and Fleury, H.J., 2009. Pol boot scanning analysis of HIV type 1 can exhibit unexpected recombinations. *AIDS Res. Hum. Retrovir.*, **25**: 713-716. <https://doi.org/10.1089/aid.2009.0025>
- Jordan, M.R., Bennett, D.E., Bertagnolio, S., Gilks, C.F., and Sutherland, D., 2008. World health organization surveys to monitor HIV drug resistance prevention and associated factors in sentinel antiretroviral treatment sites. *Antivir. Ther.*, **13**: 15-23.
- Junqueira, D.M. and de Matos Almeida, S.E., 2016. HIV-1 subtype B: Traces of a pandemic. *Virology*, **495**: 173-184. <https://doi.org/10.1016/j.virol.2016.05.003>
- Kalichman, S., Katner, H., Banas, E. and Kalichman, M., 2017. Population density and AIDS-related stigma in large-urban, small-urban, and rural communities of the southeastern USA. *Prev. Sci.*, **18**: 517-525. <https://doi.org/10.1007/s11121-017-0761-9>
- Keele, B.F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M.L., Bibollet-Ruche, F., Chen, Y., Wain, L.V., Liegeois, F. and Loul, S., 2006. Chimpanzee reservoirs of pandemic and non-pandemic HIV-1. *Science*, **313**: 523-526. <https://doi.org/10.1126/science.1126531>
- Khan, S., Rai, M.A., Khanani, M.R., Khan, M.N.

- and Ali, S.H., 2006. HIV-1 subtype A infection in a community of intravenous drug users in Pakistan. *BMC Infect. Dis.*, **6**: 1-6. <https://doi.org/10.1186/1471-2334-6-164>
- Khan, S., Zahid, M., Qureshi, M.A., Mughal, M.N. and Ujjan, I.D., 2018. HIV-1 genetic diversity, geographical linkages and antiretroviral drug resistance among individuals from Pakistan. *Arch. Virol.*, **163**: 33-40. <https://doi.org/10.1007/s00705-017-3564-1>
- Khanani, M.R., Somani, M., Rehmani, S.S., Veras, N.M., Salemi, M. and Ali, S.H., 2011. The spread of HIV in Pakistan: bridging of the epidemic between populations. *PLoS One*, **6**: e22449. <https://doi.org/10.1371/journal.pone.0022449>
- Khanani, R.M., Hafeez, A., Rab, S.M. and Rasheed, S., 1988. Human immunodeficiency virus-associated disorders in Pakistan. *AIDS Res. Hum. Retrovir.*, **4**: 149-154. <https://doi.org/10.1089/aid.1988.4.149>
- Kurbanov, F., Kondo, M., Tanaka, Y., Zalalieva, M., Giasova, G., Shima, T., Jounai, N., Yuldasheva, N., Ruzibakiev, R., Mizokami, M. and Imai, M., 2003. Human immunodeficiency virus in Uzbekistan: epidemiological and genetic analyses. *AIDS Res. Hum. Retrovir.*, **19**: 731-738. <https://doi.org/10.1089/088922203769232520>
- Lai, M.T., Feng, M., Falgout, J.P., Tawa, P., Witmer, M., DiStefano, D., Li, Y., Burch, J., Sachs, N., Lu, M. and Cauchon, E., 2014. *In vitro* characterization of MK-1439, a novel HIV-1 non nucleoside reverse transcriptase inhibitor. *Antimicrob. Agents Chemother.*, **58**: 1652-1663. <https://doi.org/10.1128/AAC.02403-13>
- Lebedev, A., Lebedeva, N., Moskaleychik, F., Pronin, A., Kazennova, E. and Bobkova, M., 2019. Human immunodeficiency virus-1 diversity in the Moscow region, Russia: Phylodynamics of the most common subtypes. *Front. Microbiol.*, **10**: 320. <https://doi.org/10.3389/fmicb.2019.00320>
- Mujeeb, S.A. and Altaf, A., 2003. The AIDS pandemic. *N. Engl. J. Med.*, **349**: 814-815. <https://doi.org/10.1056/NEJM200308213490818>
- Rana, I.A. and Bhatti, S.S., 2018. Lahore, Pakistan—Urbanization challenges and opportunities. *Cities*, **72**: 348-355. <https://doi.org/10.1016/j.cities.2017.09.014>
- Reeves, J.D., and Doms, R.W., 2002. Human immunodeficiency virus type 2. *J. Gen. Virol.*, **83**: 1253-1265. <https://doi.org/10.1099/0022-1317-83-6-1253>
- Rhee, S.Y., Gonzales, M.J., Kantor, R., Betts, B.J., Ravela, J. and Shafer, R.W., 2003. Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucl. Acids Res.*, **31**: 298-303. <https://doi.org/10.1093/nar/gkg100>
- Saad, M.D., Al-Jaify, A., Grahan, R.R., Nadai, Y., Earhart, K.C., Sanchez, J.L. and Carr, J.K., 2005. HIV type 1 strains common in Europe, Africa, and Asia cocirculate in Yemen. *AIDS Res. Hum. Retrovir.*, **21**: 644-648. <https://doi.org/10.1089/aid.2005.21.644>
- Sanders-Buell, E., Bose, M., Nasir, A., Todd, C.S., Stanekzai, M.R., Tovanabuttra, S., Scott, P.T., Strathdee, S.A., Tjaden, J., Michael, N.L. and McCutchan, F.E., 2010. Distinct circulating recombinant HIV-1 strains among injecting drug users and sex workers in Afghanistan. *AIDS Res. Hum. Retrovir.*, **26**: 605-608. <https://doi.org/10.1089/aid.2009.0226>
- Sarker, M.S., Rahman, M., Yirrell, D., Campbell, E., Rahman, A.M., Islam, L.N., and Azim, T., 2008. Molecular evidence for polyphyletic origin of human immunodeficiency virus type 1 subtype C in Bangladesh. *Virus Res.*, **135**: 89-94. <https://doi.org/10.1016/j.virusres.2008.02.010>
- Sarrami-Forooshani, R., Das, S.R., Sabahi, F., Adeli, A., Esmaeili, R., Wahren, B., Mohraz, M., Haji Abdolbaghi, M., Rasoolinejad, M., Jameel, S. and Mahboudi, F., 2006. Molecular analysis and phylogenetic characterization of HIV in Iran. *J. med. Virol.*, **78**: 853-863. <https://doi.org/10.1002/jmv.20634>
- Shah, S., Xing, H., Altaf, A., Chen, B., Liao, L., Jia, Y., Vermund, S.H. and Shao, Y., 2011. Antiretroviral drug resistance mutations among treated and treatment-naïve patients in Pakistan: diversity of the HIV type 1 pol gene in Pakistan. *AIDS Res. Hum. Retrovir.*, **12**: 1277-1282. <https://doi.org/10.1089/aid.2010.0324>
- Song, H., Giorgi, E.E., Ganusov, V.V., Cai, F., Athreya, G., Yoon, H., Carja, O., Hora, B., Hraber, P., Romero-Severson, E. and Jiang, C., 2018. Tracking HIV-1 recombination to resolve its contribution to HIV-1 evolution in natural infection. *Nat. Commun.*, **9**: 1-15. <https://doi.org/10.1038/s41467-018-04217-5>
- Tamura, K., Dudley, J., Nei, M. and Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**: 1596-1599. <https://doi.org/10.1093/molbev/msm092>
- Tongo, M., Dorfman, J.R., and Martin, D.P., 2016. High degree of HIV-1 group M (HIV-1M) genetic diversity within circulating recombinant forms:

- insight into the early events of HIV-1M evolution. *J. Virol.*, **90**: 2221-2229. <https://doi.org/10.1128/JVI.02302-15>
- Wahid, B., 2019. An update on the severe outbreak of HIV in Kot Imrana, Pakistan. *Lancet Infect. Dis.*, **19**: 241. [https://doi.org/10.1016/S1473-3099\(19\)30038-6](https://doi.org/10.1016/S1473-3099(19)30038-6)
- Wilkinson, E., Engelbrecht, S. and De Oliveira, T., 2014. Detection of transmission clusters of HIV-1 subtype C over a 21-year period in Cape Town, South Africa. *PLoS One*, **9**: e109296. <https://doi.org/10.1371/journal.pone.0109296>
- Worobey, M., Gemmel, M., Teuwen, D.E., Haselkorn, T., Kunstman, K., Bunce, M., Muyembe, J.J., Kabongo, J.M.M., Kalengayi, R.M., Van Marck, E. and Gilbert, M.T.P., 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. *Nature*, **455**: 661-664. <https://doi.org/10.1038/nature07390>
- Yaqub, S., Yaqub, T., Shabbir, M.Z., Nadeem, A., Mukhtar, N., Rehman, F.A.U., Rahman, A.U., Shahid, M.F., Ali, M., Sohail, T. and Tahir, Z., 2019. Circulation of multiple subtypes (A, G and CRFs 02_AG) of human immunodeficiency virus type 1 (HIV-1) in selected districts of Punjab province, Pakistan. *Arch. Virol.*, **164**: 3081-3087. <https://doi.org/10.1007/s00705-019-04422-7>
- Zahra, A., Saleem, M.A., Javed, H., Khan, M.A.U., Naveed, M. and Shakoori, A.R., 2021. Single Nucleotide polymorphism induces a positive selection pressure at gag-pol sites in human immunodeficiency virus favoring drug resistance mutations. *Pakistan J. Zool.*, **112**: 2021. <https://doi.org/10.17582/journal.pjz/20200525130507>
- Zaid, M., and Afzal, M.S., 2018. HIV outbreak in Pakistan. *Lancet Infect. Dis.*, **18**: 601-611. [https://doi.org/10.1016/S1473-3099\(18\)30281-0](https://doi.org/10.1016/S1473-3099(18)30281-0)
- Zhong, P., Pan, Q., Ning, Z., Xue, Y., Gong, J., Zhen, X., Zhou, L., Sheng, F., Zhang, W., Gai, J. and Cheng, H., 2007. Genetic diversity and drug resistance of human immunodeficiency virus type 1 (HIV-1) strains circulating in Shanghai. *AIDS Res. Hum. Retrovir.*, **23**: 847-856. <https://doi.org/10.1089/aid.2006.0196>