



Single Nucleotide Polymorphism Induces a Positive Selection Pressure at Gag-Pol Sites in Human Immunodeficiency Virus Favoring Drug Resistance Mutations

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ABSTRACT

Human immunodeficiency virus the retrovirus causes AIDS. HIV mainly targets the body's immune system, including the CD₄ cells, which help the immune system fight off infections. Gag-Pol are the principal viral elements in HIV-1, which cause disease initiation and progression. Here we present a comprehensive study, where both the *in vitro* and *in-silico* analyses were performed on HIV-1 from isolates of the Punjab area of Pakistan. The main objective in the current study was to study the hypervirulence of SNPs in the Gag-Pol proteins by molecular modeling approaches. Mutational analysis in our study revealed S61A, S61M, S61Y, S61G, S61Q and M90L as the most hypervirulent mutations. This induces a selection pressure and a rate of increased virulence on Gag-Pol cleavage sites. These results significantly highlight the fact that the identified SNPs possibly contribute towards a positive selection pressure contributing to the identification of novel mutations like S61A, S61M and M90L at Gag-Pol cleavage sites harboring dominant drug resistance mutations. 3D structure validation demonstrated 87.3% in Gag and 92.5% in Pol in the most favored regions. Dolutegravir, a protease inhibitor, was used for molecular docking to reveal the drug resistance sites among the isolates under investigation. However, strikingly the binding energies resulted were -7.9 kcal/mol for Pol and -5.7 kcal/mol in Gag that predicted that SNPs in the Pol gene could be one of the reasons for drug resistance and the primary site of mutations progressing the onset of infection. These computational strategies will thus help in performing large scale studies and also in the development of novel medicines for the cure of HIV-1.

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Authors' Contribution

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

Key words

Gag-Pol, Mutations, Single nucleotide polymorphism, Hypervirulence, Drug resistance

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is initiated by the human immunodeficiency virus belonging to the Retroviridae family. Human immunodeficiency virus (HIV) mainly leads to reduced immunity, provoking increased vulnerability to opportunistic infections like HCV HBV, sexually transmitted diseases and also a variety of co-infections with HIV (Chang *et al.*, 2013). HIV-1 may have originated in

African from non-human primates and transferred to humans early in the 20th century (Worobey *et al.*, 2008). HIV-1 and HIV-2 are the two main types that 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 immunodeficiency virus. On the other hand, HIV-2 seems to have its roots in Mangabey (*Cercocebus*), Gabon, and Cameroon (Reeves and Doms, 2002).

The viral genome contains genes for the regulatory proteins such as Gag, Pol, and Env in addition to accessory and regulatory proteins (tat, rev, nef, vpr, vif, vpu). Gag encodes the matrix proteins p17, spacer protein 1 p2,

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spacer peptide 2, p1 nucleocapsid protein p7, capsid protein p24, p1 and P6 protein (Freed, 1998). While, Pol encodes RT, RNase H, integrase and protease. Cleavage of viral polyproteins, reverse transcription and integration into the host genome during the life cycle of HIV is done with the help of these viral enzymes (Mushahwar, 2006). Viral Gag-Pol precursor proteins are known to play an elemental role in the viral life cycle. These proteins thus facilitate the formation of mature virus particles through proteolytic processing (Fun *et al.*, 2012). Functional variants can have both damaging and neutral effects on the structure and function of a protein. The hypervirulent nature of a protein provokes its destabilization, altering the gene regulation, thus posing a risk in maintenance of structural integrity of cells (Arshad *et al.*, 2018). To address the question of these fatal anomalies, clinical trials and cohorts involved in Gag-Pol genotypic testing would be highly beneficial for therapeutic analysis. However, computational analysis of such genes would play a significant role in revealing underlying mechanisms and understanding patterns of drug resistance and mutations. Antiretroviral therapy (ART) is the only remedy ever since the diagnosis of this fatal virus. It favors by reducing high viral loads to undetectable levels, thus recovering the immune system. But, the increased virulency in mutations promotes drug resistance to ART which causes high rise in viral load rendering disruptions of the immune system.

Pakistan is working efficiently to fight and combat this deadly disease, which has increased in a couple of years. A sudden outbreak of HIV was reported in Kot Imrana, a small village located near Sargodha in Central Punjab in March 2018 (Zaid and Afzal, 2018). In 2019 more individuals were affected, indicating the spread of the disease to about 669 (13.38%) HIV patients and manifested increased mortality (Wahid, 2019). This crisis was followed by two major HIV outbreaks in 2019 in Rotodero among infants and sudden increased cases of HIV-1 in Faisalabad, which was an alarming health catastrophe in Pakistan (Zahra *et al.*, 2019; Mir *et al.*, 2020). To date, there is not much comprehensive molecular and computational analysis of HIV-1 in Pakistan, which could provide better insights into acquisition risks, transmission and challenges to HIV treatment. This is the first study in Pakistan on HIV-1, which covers a comprehensive *in vitro* and *in-silico* analysis targeting the Gag-Pol proteins in Punjab province of Pakistan. Since little is known about HIV-1's molecular virology and bioinformatics, the present study was aimed at analyzing the two integral genes Gag-Pol of HIV-1 at molecular and computational level using various bioinformatics strategies to detect the increased virulence and deleterious effect of the mutations. Mechanisms underlying virulence and drug resistance

need to be investigated. 3D models of the Gag-Pol protein will provide better insight for future studies in HIV and may prove helpful in development of medicines for the treatment of such non-treatable anomalies caused by genomic variations.

MATERIALS AND METHODS

Sample collection

This study was done in collaboration with Punjab AIDS Control Programme (PACP). Blood samples were obtained from different treatment centers of PACP, Lahore between November 2018 to October 2019. A total of 365 participants were recruited for this study, who had a viral load exceeding 100,000 copies/ml and a first CD₄ cell count >350 cells/ μ l on the diagnosis. Antiretroviral (ART) naïve and ART therapy were included in the sample pool. A total number of 85 (23.29%) samples were confirmed with HIV-1 infection. Fifty two males (14.25%) and 30 (9.04%) females were diagnosed with progressive HIV-1 infection. Only 33 HIV-1 (0.1%) positive isolates fulfilled the quality processing criteria for this study. Recently infected were 23 and 10 were already on ART treatment. The successfully amplified samples for HIV-1 Gag and Pol were used for genotyping and phylogenetic analysis. For detailed mutational and phylogenetic analysis, only 11 isolates qualified depending upon sociodemographic criteria mentioned in Table I. Sample collection was permitted and facilitated by PACP and informed consent was taken from the enrolled subjects. Study approval was granted by the Research Ethics Committee of the University of Central Punjab (Supplementary Fig. 2A, 2B).

Immunoassays and reference laboratory tests

All the samples were centrifuged for 3 min at 12,000 rpm followed by serum separation. The screening was then performed for the presence of HIV-1 infection by rapid testing devices. A three device method was used according to the guidelines suggested by WHO for diagnosis of HIV-1 in resource-limited countries (i) Alere™ HIV Combo Rapid Test (Alere Medical Co. Ltd., Matsudo-chi, Japan), (ii) Uni-Gold HIV (Uni-Gold, Trinity Biotech, Ireland; #1206502) and (iii) SD Bioline HIV-1/2 3.0 (Standard Diagnostics, Kyonggi-do, South Korea).

CD₄ count testing and HIV detection by real-time PCR

After collection of the blood samples, 25 μ l of blood was added to the Alere PIMA™ CD₄ cartridge and CD₄ count was displayed on the Alere PIMA™. Real-Time PCR was performed on the cobas 4800 HIV-1, COBAS®

AmpliPrep/COBAS® TaqMan® HIV-1 system, an *in vitro* nucleic acid amplification test for the quantification of HIV-1 RNA in plasma following automated specimen processing, amplification and detection. The test quantitates HIV-1 RNA at a range of 20-10,000,000 copies/mL (33 to 1.67x 10⁷ International Units IU/mL) based on the WHO international standards recommended for HIV-1.

Table I. Baseline characteristics and sociodemographics of HIV-1 individuals.

Gender	
Male	7
Female	3
Transgender	1
Socioeconomic profile	
Employed	5
Unemployed	0
Students	1
Labourers	2
People with very low income	3
Predicted risk factors for infection	
Alcohol intake	0
Sharing of needles and syringes	4
Heterosexual contact	Unknown
Mother to child transmission	0
Men who have sex with Men (MSM)	Unknown
Truck drivers (travel history)	2
Unknown	5
Urban/ rural population	
People residing in cities	7
People residing in village/ suburbs	4
CD₄ count (cells/μL)	
< 200	6
200-350	5
350-500	0
>500	0

Nucleic acid extraction, reverse transcription, polymerase chain reaction and sequencing

Viral RNA was extracted from 200 μ L of plasma by QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. Revert Aid First Strand cDNA Synthesis Kit

(Thermo Scientific, USA, # K1622) was used for cDNA preparation. Nested polymerase chain reaction (PCR) was used for the amplification of *Gag* and *Pol* genes. Partial *Pol* gene containing protease (*PR*) gene of 99 amino acid codons and the first 298 codon segment of the reverse transcriptase (RT) was amplified. Both *Gag* and *Pol* fragments were amplified by nested RT-PCR the method described by [Chen *et al.* \(2014\)](#). 1% agarose gel was used for visualization of the PCR products. Successfully 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).

Computational analysis

The sequences of *Gag* and *Pol* were submitted to Genbank (accession numbers MT 462197, MT 462198, MT 462199, MT 462200, MT 489306, MT 489307, MT 489308, MT 489303, MT 489304, MT 489305, MT 462202) in FASTA format using BankIt-NCBI-NIH database. Mutational analysis for Gag-Pol was performed by POLYPHEN 2, PROVEAN, I-MUTANT, SIFT and PHD-SNP to predict the allele function, effect of non-synonymous SNPs, structure and function-based characteristics ([Adzhubei *et al.*, 2013](#); [Choi and Chan, 2015](#); [Kamaraj and Purohit, 2013](#); [Kumar *et al.*, 2012](#)). POLYPHEN 2 uses the naïve Bayesian classifier to predict the allele function with a score ranging from 0-1 calculating the probability for a given mutation to be deleterious mutation and a score nearer to 1 ([Adzhubei *et al.*, 2013](#)). The prediction of conserved domains was performed by Pfam ([Finn *et al.*, 2015](#)), CDD ([Marchler-Bauer *et al.*, 2014](#)) and Scan Prosite ([Hulo *et al.*, 2006](#)). To predict a protein sequence variation and its function, Protein Variation Effect Analyzer (PROVEAN) was used. When the score equalizes to a predefined threshold i.e. -2.5; the protein variant is found to have a more hypervirulent effect causing damage ([Capriotti *et al.*, 2006](#)). The impact of non-synonymous SNPs on the structure and function of proteins was determined by using I-MUTANT 3.0. Sorting intolerance from tolerance (SIFT) is a web-based tool using the PSI-BLAST protein database. Functionally associated protein sequences were collected using SIFT. Due to the difference in software and algorithms, their predictions may differ from each other ([Ng and Henikoff, 2001](#)). PHD-SNP and SVM based classifier, classifies substitutions at specified positions, amino acid replacements and profile-based prediction. It classifies the mutations as neutral or deleterious polymorphism ([Finn *et al.*, 2015](#)). Pfam database was used for analysis for the large collection of protein families represented by multiple sequence

alignments (MSA) and hidden Markov models (HMMs). To reveal the biomolecular interactions and mechanisms, docking methodologies are widely applied to structure-based drug design investigations. Further the visualization of the protein–ligand interactions were made by the PyMOL software. Computational docking for the study of biomolecular interactions and mechanisms was done by AutoDock Vina (AutoDock 4) (Bhargava *et al.*, 2014). Phylogenetic tree analysis was performed using the neighbor-joining method with 1000 bootstrap replicates using MEGA (Molecular Evolutionary Genetics Analysis, version 5.1) (Tamura *et al.*, 2011). RAMPAGE (Ho and Brasseur, 2005) and PROCHECK (Laskowski *et al.*, 1993) tools were used for checking stereochemical quality, analyzing overall residue-by-residue geometry.

Table II. Mutational analysis using POLYPHEN-2, PROVEAN, I-MUTANT, PHD-SNP, SIFT.

Gag gene	Mutations				
	POLYPHEN-2	PROVEAN	I-MUTANT (stability)	SIFT	PHD-SNP
A 219 P	Specific 0.112	Neutral 1.558	Decrease 8	Tolerated 0.05	Diseased 9
C263I	Specific 0.224	Deleterious -4.282	Decrease 5	Tolerated 0.10	Diseased 5
N239K	Specific 0.161	Neutral -3.456	Decrease 2	Tolerated 0.91	Diseased 2
A222G	Specific 0.977	Neutral 2.321	Decrease 10	Tolerated 0.31	Neutral 1
S61A	Sensitive 1.23	Deleterious -4.322	Decrease 6	Tolerated 0.43	Diseased 7
S61V	Specific 0.076	Deleterious -1.256	Decrease 6	Tolerated 0.89	Diseased 7
S61L	Not predicted 0.00	Neutral 1.549	Decrease 5	Tolerated 0.54	Neutral 3
S61I	Specific 0.433	Neutral 1.985	Decrease 3	Tolerated 0.65	Neutral 2
S61M	Sensitive 1.33	Deleterious -2.456	Decrease 6	Tolerated 0.67	Diseased 4
S61F	Specific 0.441	Neutral 1.890	Decrease 5	Tolerated 0.79	Diseased 2
S61W	Specific 0.569	Neutral 1.789	Decrease 6	Tolerated 0.78	Neutral 1
S61Y	Sensitive 1.78	Deleterious -2.456	Decrease 7	Tolerated 0.34	Diseased 0
S61G	Sensitive 1.27	Deleterious -4.578	Decrease 9	Tolerated 0.54	Diseased 5
S61P	Specific 0.344	Neutral 0.433	Decrease 4	Tolerated 0.73	Neutral 0
S61S	Specific 0.442	Neutral 2.654	Decrease 9	Tolerated 0.87	Neutral 5
S61T	Specific 0.554	Neutral 1.983	Decrease 8	Tolerated 0.94	Neutral 5

Gag gene	Mutations				
	POLYPHEN-2	PROVEAN	I-MUTANT (stability)	SIFT	PHD-SNP
S61C	Specific 0.221	Neutral 1.976	Decrease 5	Tolerated 0.58	Diseased 2
S61H	Specific 0.119	Diseased -0.987	Decrease 7	Tolerated 0.49	Neutral 0
S61R	Specific 0.004	Deleterious -1.432	Decrease 7	Tolerated 0.67	Diseased 5
S61K	Sensitive 1.44	Deleterious -1.987	Decrease 9	Tolerated 0.87	Diseased 0
S61Q	Not predicted 0.00	Neutral 1.566	Decrease 7	Tolerated 0.76	Neutral 1
S61E	Sensitive 1.28	Deleterious -2.334	Decrease 6	Tolerated 0.65	Diseased 1
S61N	Sensitive 1.37	Deleterious -2.987	Decrease 6	Tolerated 0.94	Diseased 2
S61D	Specific 1.34	Neutral -1.322	Decrease 5	Tolerated 0.36	Neutral 1
V81I	Sensitive 1.89	Deleterious -2.890	Increase 2.0	Not tolerated 0.98	Diseased 5
I116N	Specific 0.445	Neutral 1.876	Decrease 5	Tolerated 0.54	Diseased 0
K115Q	Specific 0.655	Neutral 2.345	Decrease 3	Tolerated 0.34	Diseased 3
M114I	Sensitive 1.34	Deleterious -1.445	Increase 3.0	Not tolerated 0.93	Diseased 4
D113E	Sensitive 1.99	Deleterious -1.322	Increase 7.0	Not tolerated 0.99	Diseased 4
G125S	Specific 0.886	Neutral -2.12	Decrease 9	Tolerated 0.26	Diseased 2
T170S	Specific 0.645	Neutral 1.987	Decrease 8	Tolerated 0.54	Neutral 4
N239T	Not predicted 0.00	Neutral 0.223	Decrease 2	Tolerated 0.67	Neutral 3
G248S	Sensitive 1.325	Deleterious -1.876	Decrease 8	Not tolerated 0.91	Diseased 1
Pol gene					
M90L	Sensitive 1.88	Deleterious -1.997	Increase 3.0	Not tolerated 0.96	Diseased 2
M143L	Specific 0.457	Deleterious -2.23	Decrease 2	Tolerated 0.31	Neutral 5
L242I	Specific 0.644	Neutral 1.739	Increase 0	Tolerated 0.49	Diseased 6
D269E	Sensitive 1.47	Neutral 1.677	Increase 5.0	Not tolerated 0.95	Neutral 3
D277E	Specific 0.876	Neutral 1.889	Decrease 7	Tolerated 0.76	Diseased 5
I278M	Not predicted 0.00	Neutral 0.999	Decrease 5	Tolerated 0.87	Diseased 0
T100I	Sensitive 1.58	Deleterious -1.028	Increase 5.0	Not tolerated 0.89	Diseased 2
D350E	Specific 0.875	Neutral 1.442	Decrease 7	Tolerated 0.76	Neutral 6
Y221H	Specific 0.710	Neutral 1.133	Decrease 8	Tolerated 0.65	Diseased 4
T160V	Sensitive 1.20	Deleterious -2.559	Decrease 6	Not tolerated 0.98	Diseased 5

RESULTS

Mutational analysis

We subjected all the detected single nucleotide polymorphisms (SNPs) of *Gag* and *Pol* to five different *in silico* approaches like POLYPHEN 2, I-MUTANT, SIFT, PROVEAN and PHD-SNP were used to investigate the effect on the structure of the Gag-Pol protein. However, four tools I-MUTANT, PHD-SNP, SIFT and PROVEAN strongly verified the prevailing hypervirulence in Gag and Pol with a higher degree of deleterious, specific and sensitive SNP values as shown in Table II. The identified SNPs demonstrated deleterious and damaging effects with the specified scores, thus depicting an increased influence of diseased SNPs shown in Table II.

POLYPHEN 2 (Polymorphism Phenotyping V2)

Almost the majority of the mutations in Polyphen 2 analysis showed a change in amino acid towards specificity and sensitivity, denoting that the mutation causes a deleterious and damaging effect with a score near to 1.00 and greater than 1 (Table II). The highest specificity was demonstrated at position S61 in Gag and M90L in Pol, giving a score of 1.88 (Table II). The mutational analysis of this research study revealed S61A, S61M, S61Y, S61G and S61Q were the most dysfunctional mutations in Gag and probably could be one of the reasons for increased drug resistance. The deleterious SNPs are based on position-specific independent count score difference, where score “1” is considered the most deleterious and our results also demonstrated synchrony as reported in some recent computational studies (Arshad *et al.*, 2018).

PROVEAN

The cutoff for Gag variants C263I, S61A, S61N, V81I and in Pol T160V depicted a significant deleterious effect of more than -2.5 thus denoting as the most virulent mutations detected by Provean analysis (Table II).

I-MUTANT

In the current study, majority of the SNPs (Table II) in the *Gag* gene, demonstrated a decreased score of greater than “5” significantly denoting the fact that the disease could be caused by any of these mutations. SNPs D269E and Y221H showed a score of about 5 and 8, respectively, in the *Pol* gene exhibiting deleterious effect with increased virulent influence. The output predicted the decreased changes in protein stability upon the possible mutations for a given position with RI value (Reliability Index) 1 and 2 for the transitions, as mentioned above. Our results revealed that most of the SNPs detected showed decreased stability in I-mutant detected Gag-Pol variants (Table II).

In accordance with these results, previous studies have also demonstrated decreased protein stability leading to degradation, misfolding and aggregation of proteins (Mayer *et al.*, 2007). This phenomenon of decreased stability, however, sheds light on the notion that these detected polymorphisms probably cause the maximum harmful effect to the protein by affecting its stability and disrupting the proper translation of the protein.

PHD-SNP

The output of Gag-Pol variants by PHD-SNP (Table II) contains mutations in the protein sequence. A219 P, a Gag variant, demonstrated mutation with a score of 9 and L242I, a Pol detected variant gave a score of 6.

Conserved domains and motifs prediction

For the prediction of conserved domains, the following tools were used:

Pfam

The domains predicted by Pfam for Gag-Pol from the 11 isolates were Gag_p17, Gag_p24, zf-CCHC, RVT-1, RVT thumb, RVT connect, RNase-H, Integrase-Zn and MLVINC as shown in Figure 1. The matrix proteins of Gag showed an E-value of 1.6e-61 and 4.8e-79, whereas Pol demonstrated an E-value of 8 i.e.e-31 and 5.6e-18 of RVT-1 and RVT thumb (Supplementary Table S1). The significant domains of Pol were protease, RNase H and Integrase, which denoted the E-values 1.1e-24, 3.3e-11 (Supplementary Table S1). These results infer the fact that the major mutations in both these genes could be in these detected domains with raised E-values suggesting a higher rate of virulency in mutations of these genes. Highly conserved residues could be functional or structural based on their location on the protein surface or inside its core (Doniger *et al.*, 2008). Amino acids involved in vital processes, for example, in interactions among different proteins, are more conserved than others. SNPs located at the conserved regions are considered immensely deleterious to protein as compared to those at non-conserved sites (Miller and Kumar, 2001).

CDD

Results of conserved data domain (CDD) revealed 10 major conserved domains named as RT-rtv, Gag_p24, Gag_p17, RVT connect, RNase- H, RVP, rve, RVT-thumb (Figs. 2A and 2B). The highest E-values were depicted by Pol region of domains RVT-1 and RVT_thumb with 8.1e-31 and 5.6e-18. These domains majorly contributed to the catalytic activity of proteins at the N-terminal. Gag_p24, mainly involved in the formation of the inner protein layer, depicted an E-value of 1.36e - 64, whereas Gag_p17, the matrix



Fig. 1. Sequence matches and features of Gag-Pol-pol as predicted by Pfam: 10 domains Gag_p17, Gag_p24, zf-CCHC, RVT-1, RVT_connect, RNase-H, RVP, RVT-thumb, MLVINC, Integrase-Zn were best predicted by Pfam as shown in the figure.

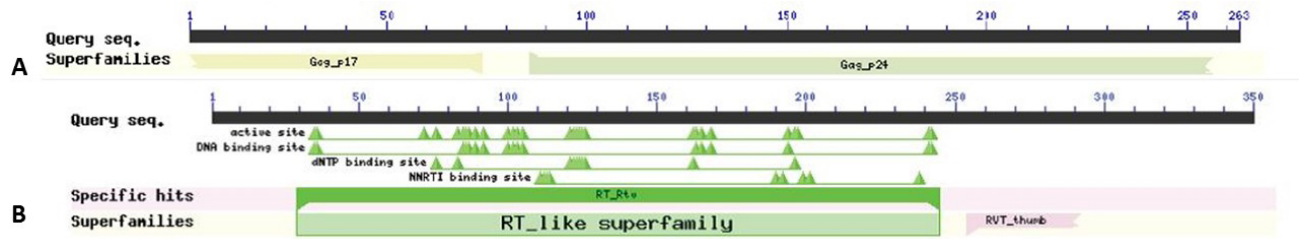


Fig. 2. A and B, Predicted conserved domains and E-values of Gag-Pol by CDD database of NCBI: Highly conserved regions of Gag_p17, Gag_p24, RVT-1, RVT_thumb were predicted in HIV-1. The domains for Pol showed an active site, DNA binding site, dNTP binding site and NNRTI binding site.

Table III. Conserved domains as predicted by CDD: Description analysis for Gag_p24 and Gag_p17 describing as core nucleocapsid and icosahedral proteins for the mature immunodeficiency virus with E-value as 1.36e-64 and 1.17e-20. RT_Rtv and RVT-thumb (four helix domain) catalyzing the major part of the virus from RNA to DNA for integration into the host depicted the major E-values as 5.51e-123 and 1.66e-12 respectively. The Interval values were 86-256, 1-74 for the matrix Gag proteins and 29-245, 254-292 for Pol proteins.

Domain	Accession ID	Description	Interval	E-value
Gag_p24 superfamily	c102908	Gag gene protein p24 (core nucleocapsid protein) p24 forms inner protein layer of the nucleocapsid. ELISA tests for p24 is the most commonly used method to demonstrate virus replication both in vivo and in vitro.	86-256	1.36e-64
Gag_p17 superfamily	c102892	The matrix protein forms an icosahedral shell associated with the inner membrane of the mature immunodeficiency virus.	1-74	1.17e-20
RT_Rtv	cd01645	Reverse transcriptases catalyze the conversion of single-stranded RNA into double-stranded viral DNA for integration into host chromosomes. Proteins contain long terminal repeats (LTRs) and are multifunctional enzymes with RNA-directed DNA polymerase, DNA directed DNA polymerase, and ribonuclease hybrid (RNase H) activities. The viral genome enters the cytoplasm as part of a nucleoprotein complex, and the process of reverse transcription generates in the cytoplasm forming a linear DNA duplex via an intricate series of steps.	29-245	5.51e-123
RVT_thumb super family	c106055	This domain is known as the thumb domain. It is composed of a four helix bundle.	254-292	1.66e-12

protein gave an E-value of 1.17e-20 (Table III). Pol gene gave an E-value of 5.51e-123 in RT_RTV and RVT thumb consisted of four-helix bundles sustains an E-value of 1.66e-12 (Figs. 2A and 2B).

Scan prosite

The predictions resulted from Scan Prosite were functional sites, protein families by comparing amino acid patterns in the Gag-Pol protein domains like ferredoxin type iron sulphur binding regions, EGF-1, Anaphylatoxin-1,

Aspartyl proteases and RT-Pol exhibited a confidence level of (-1) and increased high scores (Supplementary Table S2). These predictions reveal information about the biochemical function of the core HIV-1 proteins and have also been described previously in other studies (Naveed *et al.*, 2014).

Secondary structure prediction

The translate tool EXpasy was used to predict the secondary structure of the protein. The DNA sequence

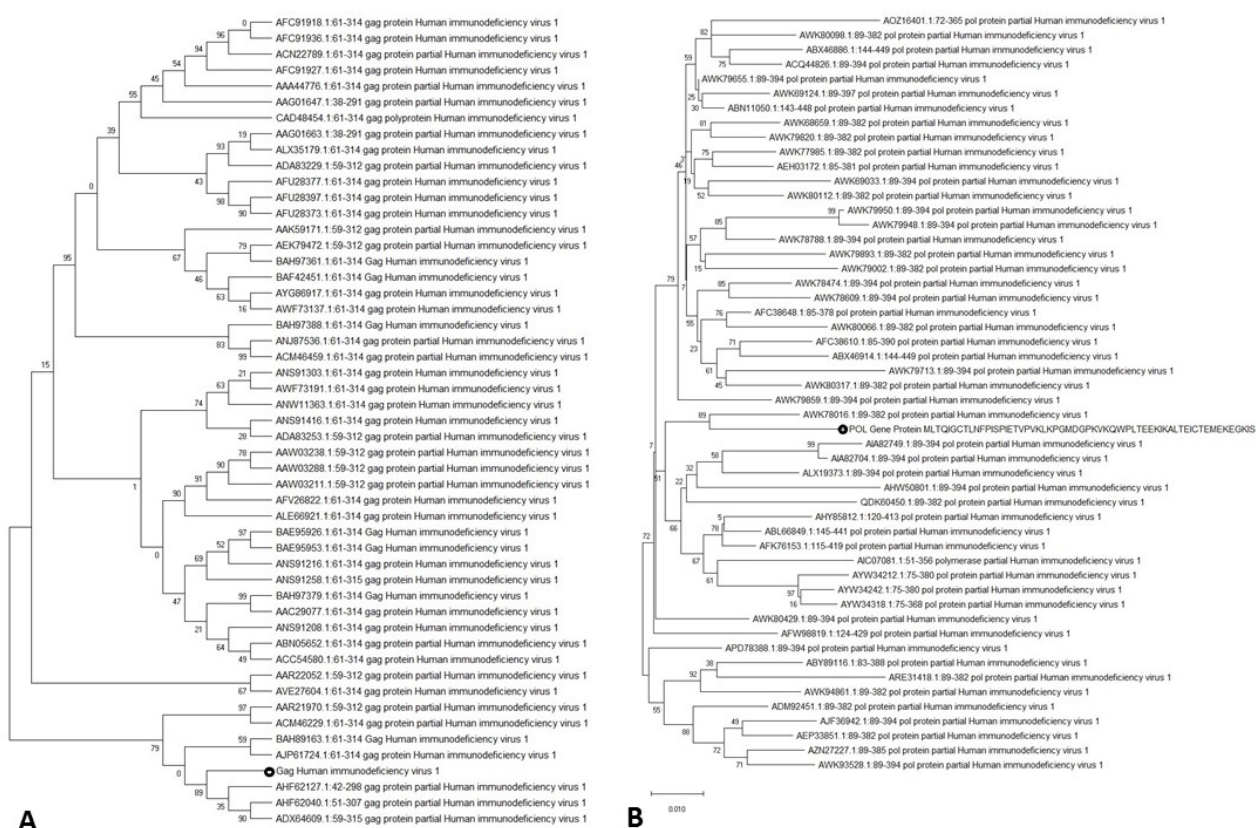


Fig. 3. Phylogenetic tree generated via Neighbor Joining method. A: shows phylogenetic tree of Gag. B: shows phylogenetic data of Pol.

was translated by EXpasy, further, the translated sequence was modeled with I-TASSER for tertiary structure prediction.

Tertiary structure prediction

The first model having the best-predicted scores gave a C-score of -1.18, TM-score 0.57 ± 0.15 and an estimated RMSD $8.6 \pm 4.5 \text{ \AA}$ in the Gag 3D predicted model (Supplementary Fig. 1A). The Pol 3D model showed a C-score of -0.49, TM-score of 0.65 ± 0.13 and an estimated RMSD $7.6 \pm 4.3 \text{ \AA}$ (Supplementary Fig. 1B). Similar studies done previously on GDH gene and mental retardation genes like TUSC3 have used I-TASSER for tertiary structure prediction (Naveed *et al.*, 2014, 2016). These findings significantly strike the notion which would be highly beneficial in the development and understanding of novel therapeutic strategies for diseases.

Phylogenetic analysis

MEGA 5 software was used to elucidate the phylogenetics and ancestral history in the current study. The

(Neighbor-Joining method) was used for the construction of a phylogenetic tree. The interior branch method and a bootstrap value of 1000 were implemented to validate the ancestral relationships (Figs. 3A and 3B).

3D Structure validation

Ramachandran plot

The 3D structure validation was done by RAMPAGE (<http://services.mbi.ucla.edu/SAVES/Ramachandran/>) and PROCHECK (Ho and Brasseur, 2005) tools which provide a view of the conformation of a protein and verification of the 3D structure. Gag had 87.3% residues in the most favored regions, 11.3% in additional allowed regions, 1.0% in generously allowed regions, 0.4% in disallowed regions (Fig. 4A). However, the final model of Pol had 87.3% residues in the most favored regions, 11.3% in additional allowed regions, 1.0% in generously allowed regions, 0.4% in disallowed regions, number of non-glycine and non-proline 100% residues. The total number of residues was 821 (Fig. 4B). Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected

to have over 90% in the most favored regions.

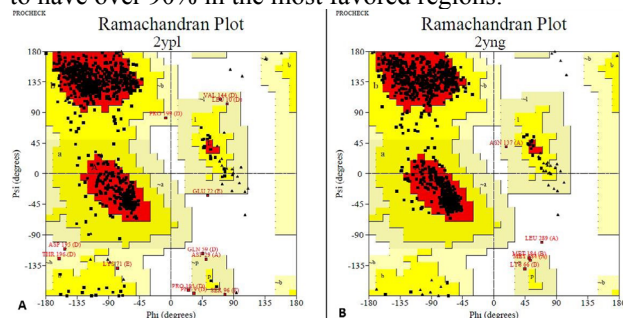


Fig. 4. Rampage analysis: The Ramachandran plots show the phi (ϕ)-psi (ψ) torsion angles for every residue of a protein. A, The final model had 87.3% residues in the most favored regions, 11.3% in additional allowed regions. Total number of residues were 821. B, The final model had 92.5% residues in the most favored regions, 6.9 % in additional allowed regions. Total number of residues were 971. Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Auto dock vina

Tools such as Pymol, discovery studio and Mgl tool were used in docking methodology (Trott and Olson, 2010). Here we checked the binding capabilities between the protein of HIV and the most recommended drugs for HIV-1 like Dolutegravir (DTG) ligand possibly for control of AIDS. Molecular docking imaging analysis (Figs. 5 and 6) exhibited interactions visualized between the protein and ligand. Structural shreds of evidence demonstrated the hydrogen bonds and binding interactions both in Figures 5 and 6, respectively. Drugs like DTG have been well known for their inhibition reported in previous and current studies. DTG mainly functions by binding the catalytic domain of the HIV Gag-Pol region, diminishing the activity of the viral enzymes by the assembly of the HIV genome ceasing into the host which blocks the access of the substrate rendering the entry to the catalytic site (Wainberg *et al.*, 2016). For the current research analysis, DTG was used as a major docking drug with the obtained I-TASSER structures of the isolates to check the ligand-binding sites and increased virulency among them and comparable with the wild type structure of HIV.

DISCUSSION

Principally in the mechanism, HIV-1 protease inhibitors (PIs) compete with the Gag substrates for attachment with the active site of the protease (da Silva *et al.*, 2019). However, even when the enzyme exhibits reduced activity, secondary mutations develop in the protease gene, which

fastens the process of Gag substrate cleavage, thus initiating

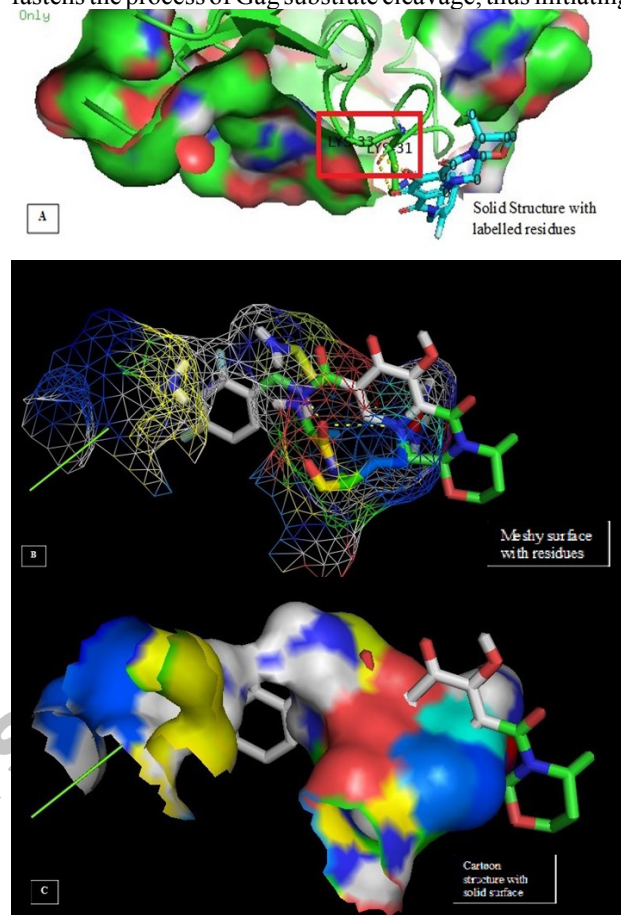


Fig. 5. Molecular docking image analysis of Gag. The interactions are visualized between the protein and ligand of Dolutegravir for Gag protein. Dolutegravir is one of the core drugs used for treatment of HIV-1 targeting the Gag-Pol region. A shows the solid structure with residues; B represents the meshy surface and C represents the solid surface cartoon.

viral fitness (Dam *et al.*, 2009). Alternative theoretical methodologies have been proposed in the literature previously suggesting that mutations in the Gag-Pol region actively participated in the resistance against protease inhibitors (PI) and that is resulting in the virus resistant to these PIs in the absence of any major mutations in the protease gene (Nijhuis *et al.*, 2007; Verheyen *et al.*, 2006). Docking results showed binding affinities of both Gag and Pol with protease inhibitors like Dolutegravir gave a binding affinity of -5.7 kcal/mol in Gag and -7.9 kcal/mol in Pol (Supplementary Table S3A and S3B). This suggests that DTG one of the most potential drug for control of HIV. However the findings of this study also gives coverage to the fact that drug resistance could be mainly in the Gag-

Pol region of HIV-1 thus suggesting that these two proteins create a positive selection pressure at Gag-Pol cleavage sites leading to progression of the disease. However, the binding affinity of DTG was lower in Pol, which suggested significantly increased virulence in the Pol region and enhanced vulnerability to mutations with an up-regulated hypervirulence. The binding sites were very few or only in a single place, as shown in Figure 5A with lysine residues and with lysine and valine residues (Fig. 6A). In addition to this, the results of docking of this study also shed light on the phenomenon that in future therapeutic implications with synthetic compounds of DTG can be introduced in a combination of other drugs as the major protease inhibitors for ART therapy for Pakistani population.

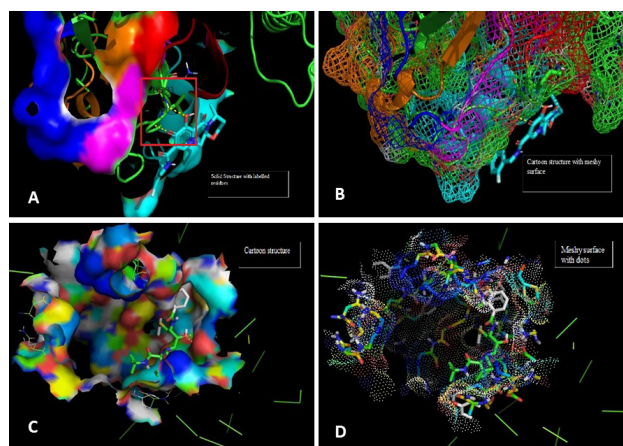


Fig. 6. Molecular docking image analysis of Pol. The interactions are visualized between the protein and ligand of Dolutegravir with Pol protein. A shows the solid surface of the protein with residue labels Lysine and valine of amino acids. B represents the cartoon structure with meshy surface depicting the binding interaction of different residues of receptor with ligand atoms as shown. In figure C the cartoon structure and binding yellow lines show the hydrogen bonds. D demonstrates the meshy surface with dots.

These *in vitro* findings confirm the molecular revelations for the major mutations and underlines the importance of Gag-Pol hypervirulence in resistance in HIV-1. As future research perspectives, this must be regarded as a primary significant phase for determining the mutational effects, positive selection pressure and interactions on viral resistance and fitness. Hence, this study can pave beneficial computational strategies for mutational analysis in the cure of HIV-1 by drug production. Computational analysis of Gag-Pol gene of Human Immunodeficiency Virus Type - 1(HIV-1) protein is useful for the identification of deleterious mutations. The binding of ligand and protein

sheds light on the phenomenon of fusion of Gag-Pol mutations leading to the initiation of AIDS. Moreover, the results of our study and computational analysis describe the suitable positions for drug designing, rendering novel methodologies for the cure of HIV. This study focuses and details the use of different bioinformatical approaches for exploring the virulent mutations, their harmful effects and structure prediction for the treatment of HIV.

CONCLUSION

Nevertheless, the underlying mechanisms still remain unclear, how HIV-1 achieves the regulation of fusion, suggesting more future studies are required to define the mechanism of this regulation. These *in vitro* findings confirm the molecular revelations for the Gag-Pol major mutations and underlines the importance of Gag-Pol mutations in resistance, very similar to the results obtained in this study. As future research perspectives, this must be regarded as a major significant phase for determining the mutational effects and interactions on viral resistance and fitness. Furthermore, such comprehensive investigations of immune diseases will help in the exploration of pathways for drug development and innovative methods for saving lives and curing HIV. Hence, this study can pave beneficial computational strategies for mutational analysis in the cure of HIV-1 by drug production.

ACKNOWLEDGMENTS

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Compliance with ethical standards

This research study was ethically approved by the Institutional Review Board of University of Central Punjab and permitted by the Punjab AIDS Control Programme also.

Funding

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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/20200525130507>

Statement of conflict of interest

The authors have declared no conflict of interest.

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Online First Article



Supplementary Material

Single Nucleotide Polymorphism Induces a Positive Selection Pressure at Gag-Pol Sites in Human Immunodeficiency Virus Favoring Drug Resistance Mutations

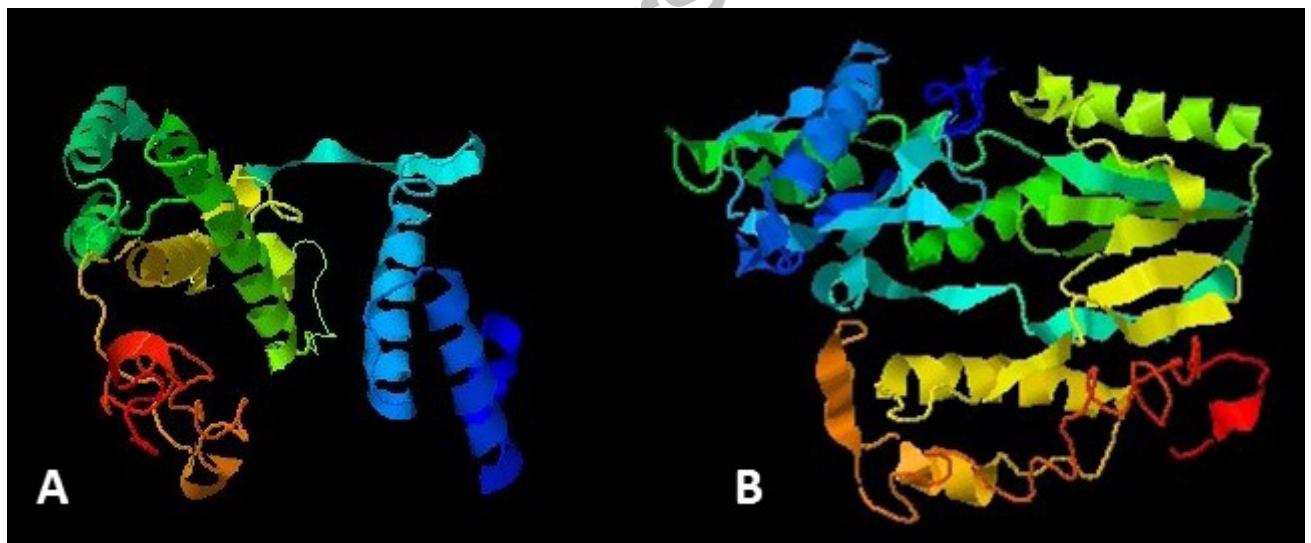
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Supplementary Fig S1 A: I-TASSER protein predicted models, A:3D view of Gag protein, B: 3D view of Pol protein

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University of Central Punjab

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April 24, 2018

The Human Research Ethics Committee
Faculty of Life Sciences
University of Central Punjab
Lahore

Subject: **REQUEST FOR THE APPROVAL OF BLOOD SAMPLE COLLECTION FOR PHD PROJECT ON MOLECULAR AND COMPUTATIONAL ANALYSIS OF HIV-1 IN PAKISTAN**

Respected Sir/Madam,

It is stated that Miss Amreen Zahra is a PhD Research scholar (Biochemistry) at Faculty of Life Sciences, University of Central Punjab under my Supervision. Her PhD research topic is **MOLECULAR AND COMPUTATIONAL ANALYSIS OF HIV-1 IN PAKISTAN**. She has successfully defended the PhD synopsis and thus will do sample collection of HIV-1 with research biotechnologist expert under complete biosafety conditions. Kindly review the project for approval in the above mentioned topic of research and your approval is highly appreciated and I express my deep gratitude and thanks for it.

Thanking you very much in anticipation.

Your's sincerely,

Supervisor:

Dr. Muhammad Naveed
Assistant Professor
FLS, UCP

Approval by the Ethics Committee:

- 1- Member 01 Dr. Muhammad Saqib Shahzad (UCP-FLS)
- 2- Member 02 Dr. Sumayya Nizamuddin (SKMCHRC)
- 3- Member 03 Dr. Muhammad Faheem (AIMC)
- 4- Member 04 Mr. Nadeem Alvi (UCP-Pharmacy)
- 5- Member 05 Mr. Muhammad Shafeeq ur Rahman (UCP-Pharmacy)
- 6- Member 06 Dr. Muhammad Naveed (UCP-FLS)

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A

PUNJAB AIDS CONTROL PROGRAM (PACP) PACP COMPLEX ADVANCED DIAGNOSTIC LAB				Document # PACP_ADL-R&D-SOP-LDOC Version PACP/V02 Issue per 10-09-2018 Effective per 15-09-2018 Pages 12/22 Author AH/QAO Reviewed By IRC Approved By PD PACP
PACP-IRC Legal Document For Conducting R&D				

PACP INSTITUTIONAL REVIEW APPLICATION FORM

University/ Institution Name UNIVERSITY OF CENTRAL PUNJAB, LAHORE

Ph No 042-35880007 Email: info@ucp.edu.pk

1. SECTION A

Project Title: <u>MOLECULAR AND COMPUTATIONAL ANALYSIS OF HIV-1 IN PAKISTAN - PHD SCHOLAR - AMREEN ZAHRA</u>			
Researchers	Names/Designation	Institutions	Signature
Principal Investigator/Supervisor (from Univ)	<u>DR. MUHAMMAD NAVEED, ASSISTANT PROFESSOR</u>	<u>FACULTY OF LIFE SCIENCES, UNIVERSITY OF CENTRAL PUNJAB LAHORE</u>	
Co-Investigator/Supervisor, if any (from Univ)	<u>DR. IRFAN AHMAD, ASSISTANT PROFESSOR</u>	<u>ALLIED HEALTH SCIENCES, UNIVERSITY OF HEALTH SCIENCES</u>	
Co-Investigator/Supervisor (from PACP)	<u>DR. HASNAIN JAVED, MOLECULAR BIOLOGIST</u>	<u>PUNJAB AIDS CONTROL PROGRAM (PACP) PRIMARY AND SECONDARY HEALTH CARE</u>	
Dates	Submission:	Review:	Protocol No.
Project involves the use of: (✓ Check all pertinent ones)			
Therapeutic drug(s)	Epidemiological Research ☒	Diagnostic research ☒	Experimental procedures Translational
Behavioral research	Community research	Radioactive agents	Other (please specify):
Full Review	Expedite Review	Exempt Review	Renewal or Modifications



B

RESTRICTED

2

Supplementary Fig. S2. Approval of blood sample collection.

Supplementary Table S1. Conserved domains prediction by Pfam: The domains predicted by Pfam of Gag-Pol region, are Gag_p17, Gag_p24, zf-CCHC RVT-1, RVT_connect, RNase-H, RVP, RVT-thumb, MLVINC and Integrase-Zn.

Source	Domain	Start	End	Gathering threshold (bits)		Score (bits)		E-value	
				Sequence	Domain	Sequence	Domain	Sequence	Domain
Pfam	Gag_p17	2	132	20.80	10.00	218.50	217.40	7.6e-62	1.6e-61
coiled_coil	n/a	87	121	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	98	131	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	104	119	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	Gag_p24	149	363	29.00	29.00	277.70	276.10	1.6e-79	4.8e-79
disorder	n/a	175	176	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	183	192	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	197	200	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	202	203	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	211	249	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	361	362	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	374	378	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	zf-CCHC	390	407	20.80	16.70	37.90	25.10	2.9e-06	0.032
Pfam	zf-CCHC	411	428	20.80	16.70	37.90	22.20	2.9e-06	0.26
coiled_coil	n/a	424	444	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	447	481	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	RVT 1	650	821	29.60	29.60	119.60	118.40	3.4e-31	8.1e-31
Pfam	RVT thumb	828	891	20.70	20.70	92.80	90.80	1.7e-18	5.6e-18
low_complexity	n/a	883	891	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	RVT connect	905	1006	21.00	21.00	197.00	196.10	1.1e-55	2.2e-55
low_complexity	n/a	982	990	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	RNase H	1023	1144	25.80	25.80	100.10	98.20	3.1e-25	1.1e-24
Pfam	Integrase Zn	1155	1192	21.70	21.70	55.70	53.80	8.6e-12	3.3e-11
disorder	n/a	1294	1296	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1298	1310	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1340	1341	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	Rve	1443	1558	32.50	32.50	92.40	90.30	5.5e-23	2.4e-22
disorder	n/a	1549	1551	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1590	1591	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1607	1611	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1617	1618	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	1619	1631	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	MLVIN C	1652	1730	29.20	29.20	116.20	115.00	1.3e-30	3.1e-30
disorder	n/a	1709	1719	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	1726	1731	n/a	n/a	n/a	n/a	n/a	n/a

Supplementary Table S2. Scanprosite results for Gag-Pol with domain descriptions.

Domain	Description	Confidence level	Range	Sequence	Score
2FE2S_FER_12Fe-2S (<i>ferredoxin-type iron-sulfur binding region signature</i>)	PS00197	(-1)	24-32	CTGATCATC	
EGF_1 (<i>EGF-like domain signature 1</i>)	PS00022	(-1)	317-328		
ANAPHYLATOXIN_1 (<i>Anaphylatoxin domain signature</i>)	PS01177	(-1)	530-562	CCtaGgtgacat.GCTctgatg...TacCat-ttgc.CC	
ANAPHYLATOXIN_1 (<i>Anaphylatoxin domain signature</i>)	PS01177	(-1)	825-855	CCagGgttaatt.GCGaacc.....TttC-cagctc.CC	
ASP_PROT_RETROV (<i>Aspartyl protease</i>)	PS50878	(-1)	16 - 85	KEALLDTGADDTVLEDINLP-GKWKPK--MIGGIGGFIKVKQY-DHILIEICGKKAIGTVLVGPTPVNIIGRNM	32.439
RT_POL (<i>Reverse transcriptase (RT) catalytic domain profile</i>)	PS50878	(-1)	139 - 329	EGKISRIGPENPYNTPIFAIKKKD-STkWRKLVDFRELNKRTQDFW-EVQLGIPHPAGLKKKKSVTVLD-VGDYFSVPLYEDFRKYTAFT-IPstnnnetpGVRVQYNVLPQGWKGS-PAIFQSSMTKILEPFRKQNpdvI-YQYMDDLVVGSDLEIGqHRTK-IEELRGHLLKWGFTTPDKKHQKEPPFLWMGYEL	48.849
ASP_PROTEASE (<i>Eukaryotic and viral aspartyl proteases active site</i>)	PS00141	(-1)	18 - 29	ALLDTGADDTVLT	0

Supplementary Table S3. Molecular docking was done to predict bound conformations and free energies of binding of small-molecule ligands to macromolecular targets. A: Shows the binding energies of Gag with Dolutegravir and the binding energies predicted were -5.7kcal/mol with distance from rmsd lower binding energy as 0.00 and best mode rmsd upper binding energies as 0.00. **B:** Shows the binding energies of pol with Dolutegravir and the binding energies predicted were -7.9kcal/mol with distance from rmsd lower binding energy as 0.00 and best mode rmsd upper binding energies as 0.00.

Mode	affinity (kcal/mol)	distance from rmsd l.b.	best mode rmsd u.b.
1	-7.9	0.000	0.000
2	-7.8	3.061	7.458
3	-7.7	9.667	13.758
4	-7.6	4.018	7.656
5	-7.1	3.347	4.479
6	-7.1	9.281	12.743
7	-7.1	5.381	7.396
8	-7.0	4.021	6.435
9	-6.9	5.399	6.658

Supplementary Table S3B. Binding affinity energies with Pol and Dolutegravir.

Mode	affinity (kcal/mol)	distance from rmsd l.b.	best mode rmsd u.b.
1	-5.7	0.000	0.000
2	-5.7	14.813	18.458
3	-5.7	13.564	17.660
4	-5.7	19.777	24.082
5	-5.6	19.700	24.163
6	-5.6	20.369	24.682
7	-5.4	19.507	23.677
8	-5.4	13.887	17.966
9	-5.4	18.641	20.808