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Research Article

Genotype Variation of HIV/HCV Co-infections in Karachi, Pakistan

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Abstract

Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) are among major health issues that affect a large section of the world's population. While HCV affects around 170 million people worldwide, HIV affects 3% of the global population, with 10 million HCV cases documented in Pakistan alone. Co-infection with HIV and HCV results in a variety of genotypic patterns, with significant global heterogeneity in the incidence of spontaneous resolution of hepatitis C and the distribution of HCV genotypes among co-infected individuals. Co-infection is usually transmitted by sexual contact, contaminated blood supplies, and mother-to-child transmission, with high-risk populations like intravenous drug users (IDUs), and men who have sex with men (MSM) having increased vulnerability. This study, conducted in Karachi, Pakistan, aimed to explore the predominant genotypes and viral load of HIV and HCV. The genetic features of the HIV *Gag* and HCV *NS5b* genes were examined using genotyping techniques and phylogenetic methodology. The study revealed that out of the 150 individuals examined, 70 were diagnosed with HIV alone, 55 with HCV only, and 25 had both HIV and HCV infections concurrently. Furthermore, genetic analysis demonstrated that HIV-1 subtype A1 was the predominant genotype, with subtype C closely following. In the case of HCV, genotype 3a was the most prevalent in both mono- and co-infected individuals. Notably, individuals with dual infections exhibited limited genotypic variability for each virus. In the case of HIV co-infection, there was an elevation in viral load, while HCV demonstrated lower viremia relative to mono-infection. In conclusion, the study revealed the predominance of HIV-1 subtype A1 and HCV genotype 3a, as well as an elevated level of HIV and a lowered level of HCV in co-infection.

Keywords: HIV infection, HCV infection, HIV/HCV co-infection, HCV genotyping, HIV genotyping.

1. Introduction

Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) infections are major global concerns. HCV is projected to infect 3% of the world's population, with 170 million people now living with the disease. The World Health Organization identifies HCV as a global epidemic, with over 10 million people in Pakistan, nearly 5% of the population, being infected. HCV replication varies in individuals' hepatocytes, leading to conditions such as chronic hepatitis, cirrhosis, and hepatocellular cancer (Hussain et al. 2011, Choo et al. 1989, Waheed et al. 2009, Abdaltif et al. 2021, Hassan 2022). Moreover, the prevalence of HCV genotypes varies by geographical area. HCV genotypes 1, 2, and 3 are common worldwide, with genotypes 1a and 1b being predominant in Japan, the United States, and Europe. Genotype 3a is prevalent among Asian nations such

as Pakistan, India, and Nepal (Tossing 2005, Zein and Persing 1996, Sohail and Rafique 2022). In North Africa and the Middle East, genotype 4 is the more commonly occurring type of HCV (Takada et al. 1993, Rehman et al. 2011), while genotype 5 is found in Hong Kong and genotype 6 in South Africa. Notably, HCV genotype 3a has been the prevalent genotype in Pakistan, causing disease in 62-70% of patients, and new data, spanning a decade, suggests its development as a novel genotype (Idrees and Riazuddin 2008, Hussain et al. 2011, Rehman et al. 2011, Sohail and Rafique 2022).

HIV and HCV share common transmission routes, including through sexual contact, exposure to infected blood or blood products, and vertical transmission from an infected mother to her child (Ogochukwu et al. 2022, Eleje et al. 2022). In Pakistan, the overall prevalence of HIV is below 0.1%, yet notably elevated among high-risk groups like intravenous drug users (IDUs) and men who have sex with men (MSM). On a global scale, HIV-1 subtype C is predominant, while subtypes B and C prevail in the Asian region (Khanani et al. 2010, Ilyas et al. 2011) However, an HIV Subtype A epidemic has been reported in an IDU group in Karachi, Pakistan (Khan et al. 2006, Bhurgri 2006, Rai et al. 2010).

HCV is five times more common than HIV-1 (Hussain et al. 2011, Waheed et al. 2009, Lauer and Walker 2001, Mohammed 2011), with prevalence varying among HIV patients depending on risk behaviors such as IDUs (60%-90%) and hemophiliacs (50%-70%), while it ranges from 4% to 8% among homosexual males(Sterling et al. 2003, Arends 2021). Co-infected IDUs continue to face a significant risk of liver-related mortality, because HIV alters HCV's normal progression, accelerating liver function deterioration (Smit et al. 2008). In HIV and HCV co-infection, HIV modifies the natural history of HCV. There is undoubted proof of accelerated liver disease progression in such patients(Rotman and Liang 2009). On the other hand, some studies have reported a strong association between HCV/HIV co-infection and increased risk of HIV disease progression (Sulkowski and Thomas 2003). Several studies have found that HCV viral load, persistence of HCV viremia, response to standard HCV therapy, and the median progression rate of hepatic fibrosis are all higher in HIV/HCV co-infected patients than in HCV mono-infection(Jamalidoust et al. 2017, Vachon, Qazi, and Dieterich 2009, Zuccalà et al. 2022). HCV viral load is a determinant of antiviral medication response and may be associated with disease spread. A significant proportion of people with HCV are also infected with HIV(Roberts and Yeung 2002, Kuniholm et al. 2011). While some evidence suggests that HCV genotype and viral load may influence the rate of HIV disease progression (Daar, Lynn, Donfield, Gomperts, O'Brien, et al. 2001, Morsica et al. 2007), other evidence suggests that HCV co-infection has little influence on the progression of HIV infection or the prognosis of those who are already infected(Morsica et al. 2007, Sulkowski et al. 2002). However, there is a lack of data on the influence of HIV and HCV on disease progression in co-infected individuals. In anticipation of a higher risk of HIV/HCV co-infection, it is critical to investigate the influence of HCV/HIV on viral load and evaluate the predominant genotypes of both HCV and HIV that co-exist in our population among both mono-infected and co-infected persons.

2. Materials and Methods

2.1. Study Population and Setting

This study was conducted at the Molecular Pathology section of Dow Diagnostics Reference and Research Laboratory (DDRRL), Dow University of Health Sciences (DUHS). The study comprised 150 individuals who were seeking HIV and/or HCV diagnoses at DUHS. Among them, 25 were co-infected with HIV and HCV, 70 with HIV alone, and 55 with HCV only.

2.2. Ethical Approval

The study was approved by the Institutional Review Board (IRB) of the DOW University of Health Sciences, Karachi, Pakistan with the IRB number: IRB-341/DUHS-12. Informed consent was obtained from all individual participants included in this study. From each patient a 10cc blood sample was collected and uniformly split between EDTA, and gel tubes. The samples were then kept at temperatures ranging from 2 to 8°C.

2.3. HIV RNA Extraction and PCR Amplification

The HIV RNA was extracted from a 140 µl plasma sample using the QIAamp Viral RNA Mini Kit

Table 1: Age and gender distribution of HIV/HCV mono and co-infected patients.

Infection	Age <20 years n= 11 (7.3%)		Age 21-40 years n=82 (54.6%)		Age >41 years n=57 (38%)		Totaln=150	
	Male 9(6%)	Female 2(1.3%)	Male 64(42.6%)	Female 18 (12%)	Male 39(26%)	Female 18(12%)	Male 112(74.7%)	Female 38(25.3%)
HIV mono-infection	8	2	34	7	17	2	59(39.3%)	11(7.3%)
HCV mono-infection	0	0	16	11	14	14	30(20%)	25(16.7%)
HCV/HIV Co-infection	1	0	14	0	8	2	23(15.3%)	2(1.3%)

Table 2: Distribution of HCV mono-infection genotype.

S. No	HCV Genotype	HCV Mono-infection
1.	1a	1(1.8%)
2.	3a	31(56.3%)
3.	3i	5(9%)
4.	4d	1(1.8%)

(QIAGEN GmbH, Hilden, Germany), according to the kit protocol. Subsequently, the isolated RNA was reverse-transcribed into cDNA using the M-MuLV RT enzyme (Thermo Scientific, USA) and Random Hexamer primers. The reaction mixture was incubated for 30 minutes at 50°C, followed by 5-minute incubation at 85°C to deactivate the reverse transcriptase (RT). The 4µl cDNA was then utilized in a nested Polymerase Chain Reaction (PCR) to amplify the HIV *Gag* gene. The first round employed G00 forward (5'-GACTAGCGGAGGCTAGAAG-3', position 761–780) and G01 reverse (5'CCAATTCCCCCTATCATTTTTGG-3', position 2264-2281) primers. For the second round of amplification, G60 forward (5'-CAGCCAAAATTACCCTATAGTGCAG-3', nt 1173-1197) and G25 reverse (5'-GCAAGTGTTTTGGCTGAAGCAAT-3', position 1867-1889 on HxB2) primers were employed. The cycling conditions for PCR amplification of the *Gag* gene included an initial denaturation at 94^{o}C for 10 minutes, followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 52^{o}C for 30 seconds, and extension at 72^{o}C for 30 seconds. The final extension was performed at 72^{o}C for 10 minutes. The amplified PCR products were separated by using 1% agarose gel, pre-mixed with 0.5µg/ml ethidium bromide, and observed under UV light before being photographed.

2.4. HCV RNA Extraction and PCR Amplification

HCV RNA was extracted from a 140 µl Plasma sample by using QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden Germany) in accordance with the protocol provided with the kit. The purified RNA was reverse transcribed into cDNA by using the M-MuLV RT enzyme (Thermo Scientific, USA) as per the manufacturer's instructions by using Random Hexamer. The reaction mixture was incubated at 50ºC for 30 minutes and followed by 5 minutes at 85ºC for RT deactivation. The PCR amplification of the HCV *NS5b* gene was done, using 4µl of complementary DNA by using forward and reverse primers for the *NS5b* gene; DM100 forward 5'-TACCTVGTCATAGCCTCCGTGAA-3' (8616–8638) and DM101 reversed 5'-TTCTCRTATGAYACCCGCTGYTTTGA-3' (8250–8275). The amplified product size was 389bp. The cycling conditions for PCR reaction of the *NS5b* gene were initial denaturation at 94^{o}C for 5 minutes followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 52^{o}C for 30 seconds, and extension at 72^{o}C for 30seconds. The final extension was performed at 72^{o}C for 10 minutes. The amplified PCR products of HCV *NS5b* were run on an agarose gel (2%) premixed with 0.5µg /ml of ethidium

Table 3: Distribution of HIV mono-infection genotype.

S. No	Genotype	HIV mono-infection
1.	A1	45 (64%)
2.	A2	1 (1.4%)
3.	B	1 (1.4%)
4.	C	16 (22.9%)
5	G	6 (8.5%)
6	J	1 (1.4%)

bromide. The products were then observed under UV light and photographed using a gel-documentation system.

2.5. HIV and HCV Viral Load

A total of 10 µl of the extracted RNA was employed for quantifying the HIV and HCV viral load on a one-step Real-time PCR (RT-PCR) reaction. This was accomplished using the commercially available artus HCV RT-PCR Kit and artus HIV-1 RT-PCR Kit (Qiagen, Germany) respectively.

2.6. Nucleotide Sequencing and Phylogenetic Analyses

Nucleotide sequences for the amplified products were determined using antisense primers for the *NS5b* gene (DM100 forward 5′-TACCTVGTCATAGCCTCCGTGAA-3′) and the *Gag* gene (G60 forward 5′-CAGCCAAAATTACCCTATAGTGCAG-3′), and the amplified product was sent to Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea). Sequenced samples were processed, trimmed, and aligned using sequence analysis software tools such as BioEdit and Mega7. The sequencing data was submitted to the National Centre for Biotechnology Information (NCBI) with accession numbers for HCV mono-infection (KY564109 to KY564146), HCV co-infection (KY564147 to KY564171), HIV mono-infection (KY445845 to KY 445914), and HIV/HCV co-infection (KY662011 to KY 662035). Reference sequences for HIV and HCV were obtained from the Los Alamos National Laboratory database (http://www.hiv.lanl.gov, http://www.hcv.lanl.gov). Phylogenetic analysis was conducted on the nucleotide sequences of the HIV-1 *Gag* gene and HCV *NS5b* gene, along with their respective reference sequences through the method, mentioned previously by (Amir et al. 2021). Phylogenetic trees were reconstructed using MEGA 7 through the Maximum Likelihood method. Bootstrap values were derived from 1000 replicate trees, and the Phylogram output option was utilized for visualization.

2.7. Statistical Analyses

Following data entry, a descriptive statistical analysis was conducted on various variables to determine frequencies, percentages, and cross-tabulation using the Statistical Package for Social Sciences (SPSS) version 17.

3. Results

Out of the 150 recruited patients, 11 (7.3%) were aged below 20 years, 82 (54.6%) were in the 21-40 years age group, and 57 (38%) were above 40 years. The gender distribution included 112 (74.7%) males and 38 (25.3%) females. Among the patients, 70 (46.6%) were solely infected with HIV, 55 (36.7%) were exclusively infected with HCV, and 25 (16.6%) were co-infected with both HIV and HCV, indicating the simultaneous presence of the two viruses in these individuals **(Table 1).**

In the phylogenetic analyses of 70 HIV mono-infected patients, 41 (58.6%) of the sequences closely clustered with the reference sequence of HIV-genotype A1. Additionally, 1 patient (1.4%) exhibited HIV genotype AE, 2 (2.9%) had HIV genotype AG, 19 (22.9%) were associated with HIV genotype C, 1 patient (1.4%) showed HIV genotype CG, 1 patient (1.4%) was identified with HIV genotype D, and 5 (7.1%) had HIV genotype G. For the 55 patients with HCV mono-infection, 31 (56.3%) were infected with HCV genotype 3a, 3 (5.5%) with 3b, 1 (1.8%) with genotype 1a, 1 (1.8%) with 3g, 1 (1.8%) with 3k, and 1 (1.8%) with 4d. Unfortunately, technical errors rendered 17 HCV sequences from mono-infected patients unanalysable (Table 2 & Figures 1 & 2).

In the genotype analyses of twenty-five HIV/HCV co-infected patients through phylogenetic reconstruction, 8 (32%) tested positive for HIV genotype A1. Among these 8 patients, 8 (32%) were

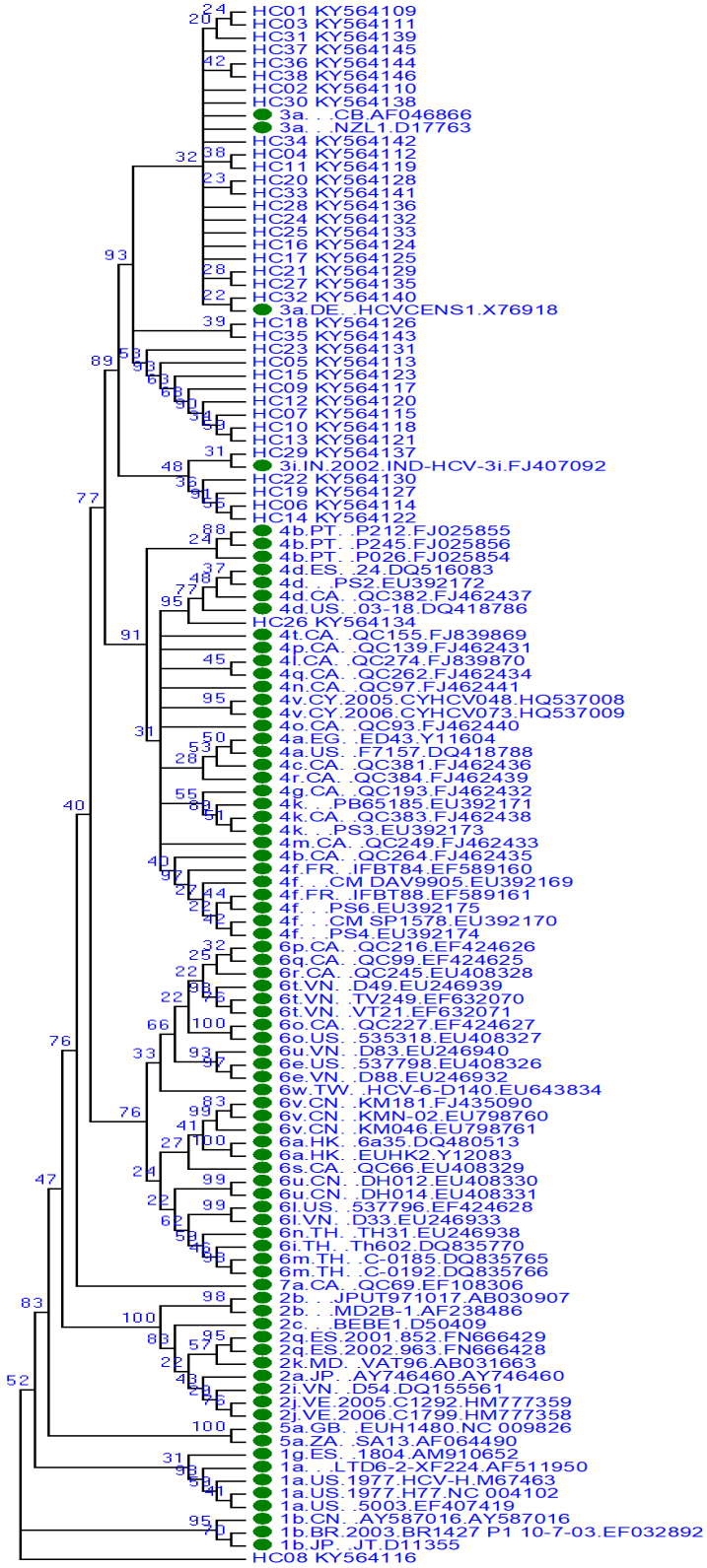

Figure 1: Phylogenetic tree of HCV sequences from mono infection with reference sequences [Maximum Like Hood method. Bootstrap values were based on the generation of 1000 replicate trees].

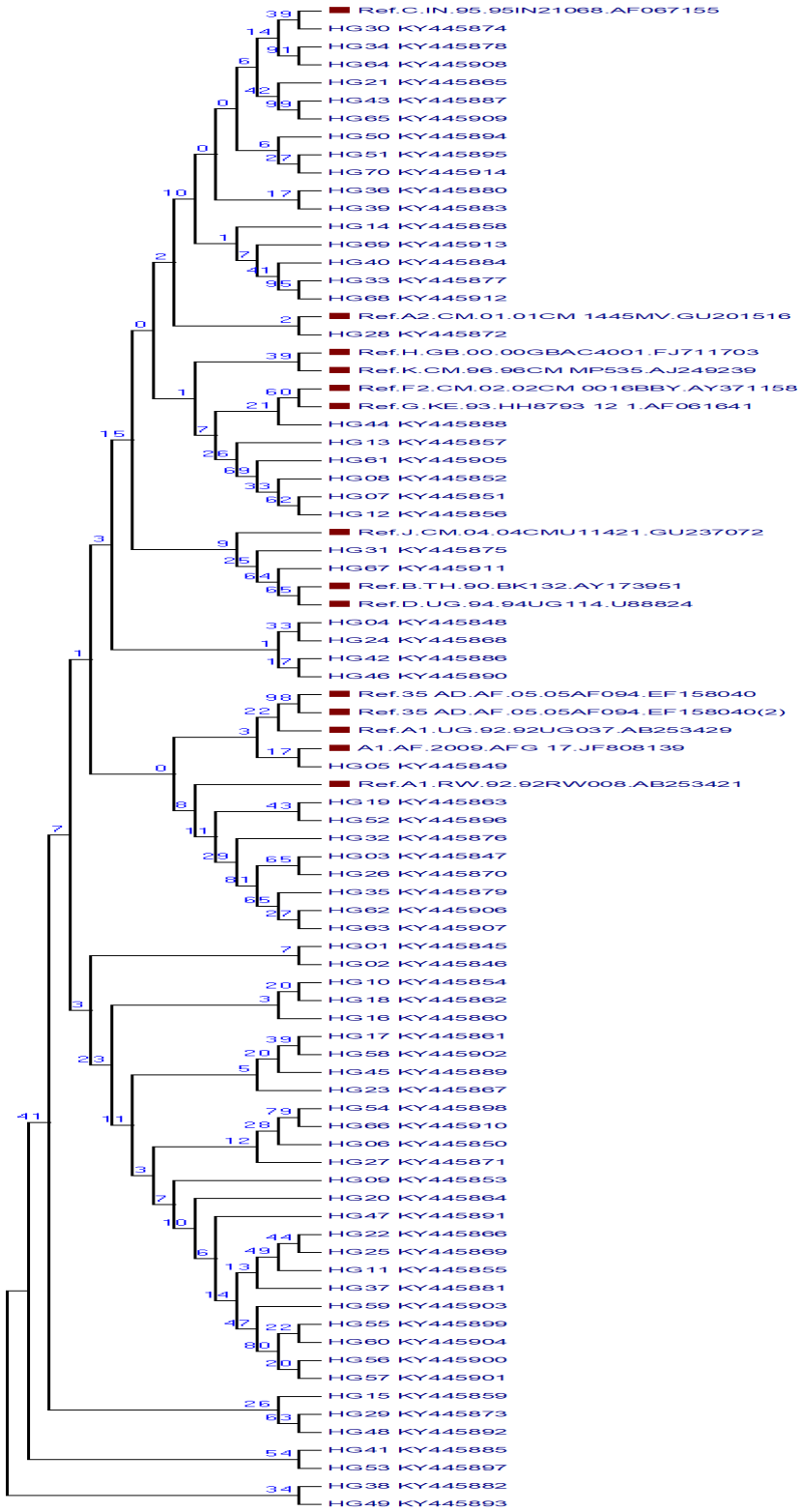

Figure 2: Phylogenetic tree of HIV sequences from mono-infected patients with reference sequences [Maximum Like Hood method. Bootstrap values were based on the generation of 1000 replicate trees].

Table 4: Cross-tabulation of HCV and HIV co-circulate in patients.

		HIV Genotype				Total
		A1	C	D	G	
HCV Genotype	3a	13(52%)	6(24%)	0	3(12%)	22
	3i	1(4%)	1(4%)	1(4%)	0	3
	Total	14(56%)	7(28%)	1(4%)	3(12%)	25

co-infected with HCV genotype 3a. In 7 (28%) co-infected patients, HIV genotype C was present, and of these, HCV genotype 3a was observed in 6 (24%) patients, while 1 (4%) case exhibited HCV genotype 3g. One (4%) HIV/HCV co-infected patient displayed HIV genotype D with HCV genotype 3b, and in 3 (12%) cases, HIV genotype G was identified alongside HCV genotype 3a (Table 4 and Figures 3-4).

The comparison of viral loads between HIV and HCV mono-infected patients and those co-infected with HIV/HCV demonstrates more efficient replication of HCV, with a median viral load of 358,721 IU/ml, compared to co-infection, which has a median HIV viral load of 190,035 IU/ml (Table 5).

4. Discussion

HIV-positive individuals are at a higher risk of co-infection with HIV/HCV due to shared modes of transmission. The targeted genomic regions for analysis were the *Gag* gene of HIV-1 and the *NS5b* gene of HCV, both crucial for the diagnosis, management, and treatment of patients. The HIV-1 Gag protein, a conserved polyprotein weighing 55 kDa, plays a significant role; polymorphisms in the Gag protein have a pronounced impact on protease activity (Goodenow et al. 2002, Mills and Jones 1990, Marcelino 2022). The p24 component of the Gag protein proves valuable in early diagnosis and is recommended as a specific target for antiviral therapeutic strategies (Gupta et al. 2001, Sutthent et al. 2003). Its component is utilized as a target in 4th generation diagnostic kits and is also an integral part of HIV multi-component vaccines. (Fox, Dunn, and O'Shea 2011, Donayre-Torres et al. 2009, Coleman et al. 2005). NS5b is a viral polymerase, also known as RNA-dependent RNA polymerase that lacks a proofreading ability, resulting in an infected individual with a population of closely related viral quasi-species (Cheney et al. 2002). NS5b plays a critical role in HCV replication, and mutations in the *NS5B* gene can impact both viral replication and the diversity of viral quasi-species. This factor serves as a significant predictor for the progression of liver disease and treatment outcomes (Qin et al. 2005, Bukh, Miller, and Purcell 1995). The sequencing and phylogenetic analysis of HCV, utilizing specific PCR-amplified genomic products, is regarded as the gold standard and is considered a conclusive method of analysis (Simmonds 1995, Stumpf and Pybus 2002). In molecular epidemiology, a phylogenetic approach is a basic tool and helps to determine the evolutionary relationships and genetic distance among the groups of organisms (Nafees et al. 2011, Parveen et al. 2021). Based on our results from 150 patients, 112 (75%) were males, and 38 (25%) were females. Among male patients, the majority, 82 (54.6%), fell within the 21-40 age range. A similar age preponderance has been noted in previous studies (Ilyas et al. 2011, Nafees et al. 2011, Khan, Ali, and Awan 2013). In this study, 70 (46.6%) patients were identified as HIV mono-infected, 55 (36.7%) as HCV mono-infected, and 25 (16.6%) had HIV/HCV co-infection. The prevalence of HCV/HIV co-infection varies among different risk groups. This variation reflects differences in behavioral patterns among diverse populations. (Khan, Ali, and Awan 2013, Ilyas et al. 2011, Ponamgi et al. 2009). The overall prevalence of HIV in Pakistan is < 1% and HCV prevalence is about 5% (Hussain et al. 2011, Safdar, Mehmood, and Abbas 2009). In the second group of HIV surveillance studies in high-risk groups, HIV prevalence amongst IDUs was 9% in 2005-2006, 15.8% in 2006-2007, and 20% in 2007-2008(Khan et al. 2006, Rai et al. 2007). Moreover, sex workers were the 2nd most high-risk population for transmission of HIV, and prevalence among transgender people (hijras) was about 4% (Rai et al. 2007). In earlier studies, HIV/HCV co-infection

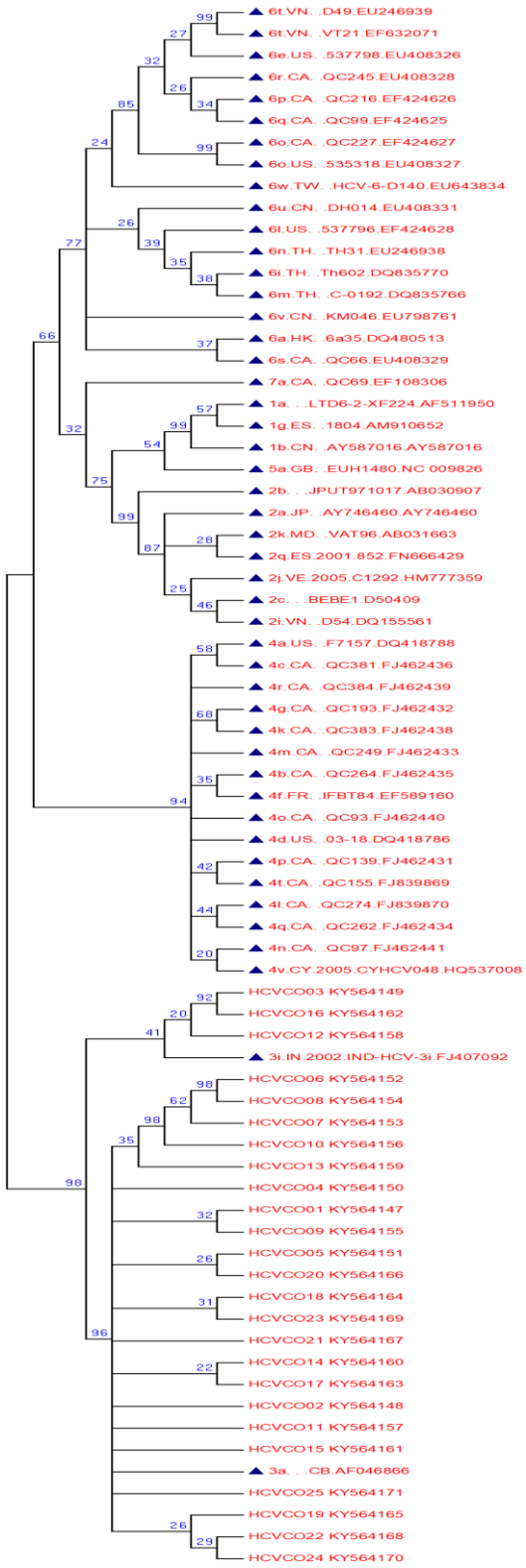

Figure 3: Phylogenetic tree of HCV Sequences from HIV/HCV co-infected Patients with reference Sequences [Maximum Like Hood method. Bootstrap values were based on the generation of 1000 replicate trees].

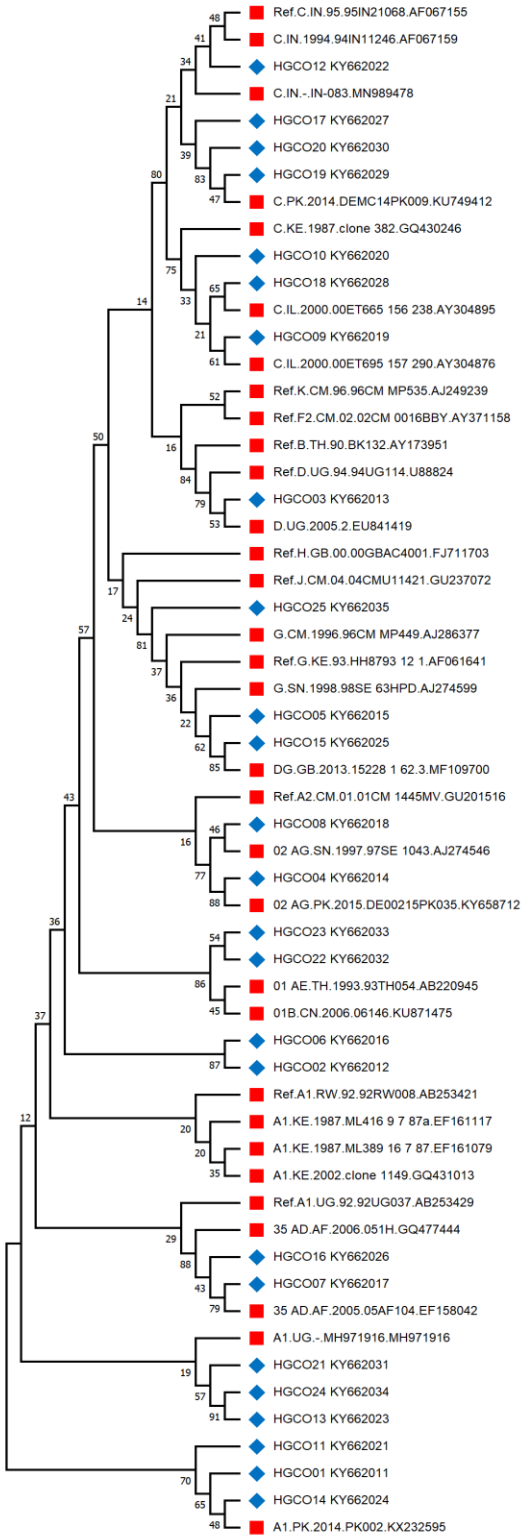

Figure 4: Phylogenetic tree of HIV Sequences from HIV/HCV co-infected Patients with reference Sequences [Maximum Like Hood method. Bootstrap values were based on the generation of 1000 replicate trees].

among Afghan refugees was 3.6%, and in MSM was 5% in Karachi, Pakistan(Khanani et al. 2010, Akhtar et al. 2015). A study conducted in Khyber Pakhtunkhwa province reported a 6.7% prevalence of HIV/HCV co-infection (Khan, Ali, and Awan 2013, Shah et al. 2015). A very high prevalence of HIV/HCV co-infection was reported in jail inmates of Sindh (90%) and Lahore (73%)(Nafees et al. 2011, Rotman and Liang 2009). A recent study in Lahore found that 32.1% of patients were positive for HIV/HCV co-infection (Ashraf et al. 2015).

In the present study, we found that there were 64% cases of mono-infection and 56% cases of co-infection with HIV-1 subtype A1, this clustered closely with those circulating in Africa. Previous studies also reported that HIV-1 subtype A1 was the most predominant subtype, and clustered with African countries, Uganda and Senegal(Bhurgri 2006,). We also found HIV-1 subtype C in 33% of cases of mono-infection and 28% of co-infection patients, which closely clustered with Indian sequences. Although, in previous studies, Indian sequences were different from sequences found in Pakistan (Bhurgri 2006). In Karachi, Pakistan major part of the population migrated from India and have relatives in India which may be a reason for frequent visits to and from across the countries. In India, the prevalent genotype is HIV-1 subtype C (Kandathil et al. 2005). We also found HCV genotype 3a in 82% of mono-infected and 88% of co-infected patients, this clustered closely with those circulating in Afghanistan and India. In previous studies in Pakistan, the HCV genotype 3 was about 85%, and subtype 3a was also reported which closely relates to our findings (Hussain et al. 2011, Idrees and Riazuddin 2008). The most important finding of our study was that 52% of patients co-circulate HIV-1 Subtype A1 and HCV genotype 3a, and 24% of patients co-circulate HIV-1 subtype C and HCV genotype 3a. However, a prior study carried out in India discovered that among patients with HIV and HCV co-infection, HCV genotype 1b was the most common genotype, followed by 1a, 3a, and 3b (Ponamgi et al. 2009, Shah et al. 2021). In China, the prevalent HCV genotypes were found to be 6a, 3b, and 1a(Garten et al. 2005, Wang et al. 2021).

The co-infection of HIV and HCV has a clinically significant early impact on immunological, and virological aspects throughout several disease phases(Operskalski and Kovacs 2011). The results of this study show that the co-inion with HCV significantly affects the HIV-RNA load, which is related to increased HIV viremia. This observation is consistent with previous studies on the subject (Morsica et al. 2007). The higher viral load in HIV/HCV co-infected individuals compared to mono-infected patients may be due to the synergistic effect of dual infections, which leads to increased immune activation and viral replication(Chen, Feeney, and Chung 2014, Osinusi et al. 2015). Furthermore, in the presence of HIV, HCV-induced immune modulation and decreased immunological control may contribute to the observed increased viral levels(Sulkowski 2008). Our findings are consistent with previous investigations that found higher HCV RNA levels in persons with HCV mono-infection, as opposed to co-infected patients who had reduced viremia or no detectable change (Daar, Lynn, Donfield, Gomperts, Hilgartner, et al. 2001, Torre et al. 2001). However, previous investigation found significantly higher HCV RNA loads in people with HIV/HCV co-infection than in people with HCV mono-infection (Berger et al. 1996, Operskalski et al. 2008, Jamalidoust et al. 2017). Although HCV and HIV individually may not significantly impact viral replication, the interaction of various factors, including antiretroviral therapy (ART), Human leukocyte antigen (HLA) Typing and virus genotypes, is observed to influence both viral replication and suppression(Kuniholm et al. 2011, Behzadpour et al. 2016, Gobran, Ancuta, and Shoukry 2021). These findings provide important insights for public health strategies and interventions targeted to the unique dynamics of HIV and HCV co-infection in Karachi, Pakistan. More study and continuing observation will be required to gain a thorough knowledge and effective control of this complex disease.

5. Conclusions

The study revealed that HIV subtype A is the predominant genotype in the population, and the identification of HIV-1 subtype C is a significant finding. HCV genotype 3a is prevalent among co-infected patients, with a majority being male and younger. Moreover, HIV-1 subtype A1 aligned

Table 5: The median viral loads in HIV/HCV mono and co-infected patients.

HIV mono-infection	**145,525 IU/ml**
HIV coinfection	190,035 IU/ml
HCV mono-infection	2,140,831 IU/ml
HCV coinfection	358,721 IU/ml

closely with African strains, while HIV-1 subtype C resembled strains found in India. HCV genotypes 3a and 3i clustered with those from Afghanistan and India. Co-infected individuals exhibited elevated HIV levels and decreased HCV levels. These findings provide valuable insights into the dynamics of HIV/HCV co-infection in Karachi, Pakistan, which has the potential to shape informed healthcare strategies.

Conflict of Interest

The authors declare that they have no competing interests.

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This research received no grant from any funding agency in the public, commercial, or not-for-profit sectors.

Study Approval

The study was approved by the Institutional Review Board (IRB) of the DOW University of Health Sciences, Karachi, Pakistan with the IRB number: IRB-341/DUHS-12.

Consent Forms

Patients signed informed consents which are available with the corresponding author.

Data Availability

All the raw data related to this study is available with the authors.

Author's Contribution

This study was conceptualized by SK, AIK, MRK, experimentation by SK, AIK, software by SK, AIK, validation by SK, MZ, and BAK, formal analysis by SK, AIK, MAQ, and AL, biochemical investigations by SK, and AIK, resources were provided by SK. data curation was performed by SK, AIK, MZ, and BAK, the original draft was prepared by SK, AIK, final draft, review and editing was done by MAQ, MSQ, IKU, IIA, ASK, visualization was done by SK, AIK, and the project was supervised by SK and MRK.

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