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Original Researh Article ***Open Access***

MOLECULAR IDENTIFICATION OF HCV GENOTYPES AMONG INJECTING DRUG USERS HAVING HCV/HIV CO-INFECTION

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Abstract *Co-infection with hepatitis C virus (HCV) and Human immunodeficiency virus (HIV) is common in Injecting drug users (IDUs). The aim of this study was the molecular identification of HCV genotypes in IDUs having HC/HIV co-infection in Peshawar. A cohort cross-sectional study was conducted in Nai Zindagi NGO from 2020 to 2022. A sample of 350 IDUs including 309 males, 23 females, 09 children, and 09 transgender were enrolled. Suspected age was 34 years. Screening of HIV and HCV infection was performed through ICT and RT-PCR. For genotype determination, a specific SACACE real-time PCR kit was used. Out of a total of 350 patients, 204 were HCV/HIV co-infected. According to bivariate analysis, there is statistically moderate positive r=522 between viral load and HCV/HIV co-infection (p=0.000). It is concluded that the prevalence of HCV/HIV co-infection was 44.28% in IDUs with the prevalent genotype 3a (51.1%). Viral load of males was higher than females. To overcome the burden of HCV/HIV co-infection large-scale, multicentre, and multistate studies should be conducted across Pakistan and preventive measures should be taken to reduce the use of syringes, razors, tattooing, sex workers, and blood transfusion.*

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Keywords: *HCV/HIV co-infection, HCV, HIV, Genotypes, viral load, RT-PCR*

Introduction

Injecting drug users (IDUs) are liable to numerous blood-borne diseases. It is evaluated that there are approximately 13 million IDUs worldwide, out of which 67% were infected with hepatitis C virus (HCV) 13% with human immunodeficiency virus (HIV) and 8.4% are living with HCV/HIV co-infection (Jamalidoust et al., 2017). HCV is a single-stranded, positive sense-enveloped RNA virus of the family *Flaviviridae*. Its diameter is about 55- 65nm. This virus causes Hepatitis C and cancers such as lymphomas and liver cancer or hepatocellular carcinoma (HCC) in humans (Ferri et al., 2015). HCV can be transferred from infected people to healthy people through different routes i.e. toothbrushes, contaminated syringes, sharing of razors, surgical equipment, barber shops, unsafe sex, contaminated dental apparatus, blood transfusion, and drug abuse. In Pakistan due to a shortage of appropriate screening of blood, untrained medical staff, and poor infrastructure, contaminated blood transfusion is the chief source of HCV transmission (Waqas et al., 2015). For HCV diagnosis and treatment, World Health Organization (WHO) has determined a goal, which is a step towards eradication of HCV by 2030. However, standards of HCV testing, diagnosis, and treatment are still low in various places all over the world. HCV has been defined as a mute epidemic, with approximately suggesting that 50% of those HCV-positive do not even know about their HCV status. HCV prevalence in people who inject drugs (PWIDs) is estimated at over 50%, than other people (Karimi et al., 2020). According to recent studies, approximately 100 million people are HCV-infected globally, and each year 700,000 deaths are caused by HCV (Lanini et al., 2016). In South Asia, around 50 million people are HCV-infected. With about 10 million chronically HCV-positive carriers, Pakistan is one of the overburdened countries. The genome of HCV encodes 7 non-structural and 3 structural genes. During replication, the viral RNA-dependent RNA polymerase initiates the formation of a genetic variant, due to a lack of proofreading mechanism and RNA nature(Khan et al., 2017). HCV has broad genetic variability, with 7 genotypes and 67 subtypes. Of all HCV infections, genotype 1 is the

most prevailing genotype which accounts for 44-46%, after that genotype 3 is (22-25%) and genotype 4 is (13-15%) (Blach et al., 2017). Geographically as well as by various population subgroups wise prevalence of genotypes is different. Of all HCV infections, genotype 1 accounts for about > 60% in Central and Eastern Europe, and genotype 3 is most common in IDUs (Baliashvili et al., 2022).

Genotype 3 accounts for more than 75% in South Asia. Genotypes 2 and 6 are endemic in East Asia. Genotype 5 is very uncommon but the majority of cases exist in Eastern and Southern Africa. Genotype 1 accounts for most cases in Middle Eastern non-Arab countries including Iran, Turkey, Cyprus, and Israel, while genotype 1 and genotype 4 are endemic in Arabian countries (Haqqi et al., 2019). In Pakistan, HCV genotype 3 (3a and 3b) is the most prevalent after the 1a, 2a, and untypable genotypes. In Punjab, Sindh, and Khyber Pakhtunkhwa (KP) genotypes 3a and 3b were proved to be highly predominant, while in Baluchistan genotypes 1a and 2a are the most prevalent (Afzal et al., 2016). In Pakistan, HCV is extremely prevalent, with about 6.8% of over-all population being HCV infected. Almost 6% of Pakistanis are actively HCV-infected. As reported by the United Nations Office on Drugs and Crime (UNODC), in the last year around 6-7 million Pakistanis were allowed to use drugs, of which around half a million were regular IDUs (Umer and Iqbal, 2016).

HIV is also a single-stranded, positive sense and an enveloped RNA virus about 120nm in diameter, a member of the Genus *Lentivirus* and family *Retroviridae*, It attacks the human immune system. Because of the high genetic variability of HIV, no significant treatment or vaccine is available against HIV infection. Over the years, HIV caused Acquired Immuno Deficiency Syndrome (AIDS). HIV/AIDS is a continuous and disastrous health issue globally, more than 36 million population are HIV infected, and over 39 million HIV/AIDS associated deaths to date. Each year about 2 million people are newly reported with HIV(Pandey and Galvani, 2019). In Pakistan, about 9 million individuals are drug addicts of which 10% are the IDUs. According to 2012 reports, in Pakistan, the ratio of HIV in IDUs was around 21% (Zahra et al., 2022). According to WHO recent reports, approximately 2.3 million individuals are HCV/HIV co-infected and more than 1.3 million HCV/HIV co-infected people are IDUs. The most frequently used drugs are heroin, amphetamines, cocaine, and other opiates. Of total heroin users, the percentage of heroin injectors rose from less than 2% in 1993 to more than 25% in 2007. In Pakistan, the frequency of sharing syringes between IDUs is 73% which is very highly correlated with the rest of the globe (Mansha et al., 2017). The association between HCV and HIV co-infection influences the natural history and transmission of HCV. In the existence of HIV infection, the transmission ability of HCV increases, with perinatal transmission risk increasing in mothers having HIV infection. Without treatment, HIV infected people have fewer chances to clear HCV infection automatically, have high HCV viral loads, and have faster HCV progression than HIV-negative people. However, ART upgrades result in HCV co-infected people, with reduced HCV associated mortality. HCV co-infection efficacy also expands the treatment of HIV infection with few proof offering a high risk of drug-associated hepatotoxicity in those people getting ART. HCV therapy has been changed with the coming of direct-acting antivirals (DAAs), which provide high cure rates within 12–24 weeks (Platt et al., 2016).

Materials and methods

Study Design

The current study was conducted in the Pathology Department of Lady Reading Hospital (LRH) with the help of HIV/AIDs control program in Peshawar.

Sample Collection

A total of 350 blood samples were collected from admitted and non-admitted IDUs patients. Sampling was done by non-probability convenient sampling technique and sample size was determined according to WHO software. All IDUs having HCV/HIV co-infection of any age of male, female, and transgender were included in the study. Patients fulfilling the inclusion criteria were admitted and non-admitted in the LRH, Peshawar. After informed consent, they were screened for HIV antibodies, HCV antibodies, and HCV genotype. From each patient 6ml blood was collected under sterile conditions in a Gel tube and EDTA tube. In the first step samples were properly labeled and screened by ICT and then ELISA using the microplate's kits. In the last step, the ELISA positive cases were confirmed through real-time PCR.

Sample Inclusion and Exclusion Criteria

Only injecting drug users suspected positive patients in the age group <20 to 61-80 years were included in the study while non-drug user patients were excluded from the study.

Sample Processing

From all drug users (2cc) blood samples were obtained and the proforma for each individual was filled. For confirmation, the samples were delivered for serological tests.

RT-PCR

In RT-PCR, an RNA was converted to cDNA by reverse transcriptase (RT), and then cDNA is amplified by the PCR. Reverse transcription provides cDNA templates for PCR amplification and downstream experiments, it is one of the most critical steps for experimental success.

PCR for HIV

In this process from patient's blood, mRNA was extracted and transformed into cDNA with the help of reverse transcriptase. PCR was performed by using specific Long Terminal Region (LTR) primers of HIV, according to Geneproof HIV type1 PCR Kit

which targets LTR sequence and GaG gene. Finally, the recognition of DNA products was processed with the help of Enzyme Linked Oligonucleotide Assay(Stevens et al., 2008).

RNA Extraction

According to the manufacturer's protocol, RNA was extracted from serum by using RNA extraction kit (Favorgene Viral Nucleic Acid Extraction Kit I, Taiwan, Cat. No. FAVNK 001-2). The extracted RNA was stored at -70°C until use (Ullah et al., 2020).

Viral Load of HCV/HIV

Viral load assessment was done by the Taqman Real Time PCR system. HCV viral load was performed by using the light cycler Taqman Master Mix kit (Roche Diagnostic GmbH, Mannheim, Germany) on Roche Light cycler version 2.0. Each sample was assessed in duplicate and the mean value was reported as the viraemic level in the serum. The unit of the HCV RNA analysis was copies/ml. In HCV RNA analysis assay, a total of 7 quality of different copy numbers was used. The range of the quality used in quantitative analysis was 10^2 to 10^8 copies/ml. In each preparation of HCV RNA, a specified quantity of internal standard was used. RT-PCR was done in the 5' untranslated region (5'UTR) according to the manufacturer's instructions (95°C for 20 sec and this was followed by a further 40 cycles at 95°C for 10 sec and 58°C for 15 sec and 10 sec at 72°C)(Chakravarti et al., 2011).

Determination of HCV Genotypes

Sacace real-time PCR-based type genotype kit (Sacace, Italy) was used. For each sample genotyping was performed according to the manufacturer's instructions. Master Mix was prepared from reagents (RT-mix1FRT, RT-Mix11, Taq polymerase) as shown in (Table 1). All ingredients were mixed and added to a specific PCR tube, 15 uL master mix along with 10uL extracted RNA (total volume was equal to 25uL). Similarly, 10uL controls, HCV genotype 1, 2, 3, and dH2O were added to the tubes marked as positive and negative control separately, and then centrifuged slowly. All PCR tubes were fixed in a PCR machine, Smart Cycler (Cepheid, USA). Adjust thermal protocols for HCV genotypes and then start running. After two hours the results were achieved(Mahmood et al., 2018).

Table 1. Master Mix for genotyping

Reagents	Volume in uL/Sample
RT-Mix 1 FRT	10
RT-Mix 11	0.5
Taq polymerase	0.5
Total	15.5

Statistical analysis

Statistical analysis was performed by using SPSS version 23. One-way ANOVA test was performed for significance (p-value). Pearson's correlation (r) was used for positive and negative performance.

Results

Demographic Analysis

During this cohort cross-sectional study 350 samples were collected from different genders including 309/350 (88.3%) cases of male, 23/350 (6.6%) cases of female, 09/350 (2.6%) cases of children, 09/350 (2.6%) cases of transgender were studied. Out of 350 samples of IDUs, 66/350 (18.9%) individuals were positive for HCV mono-infection, 23/350 (6.6%) individuals have HIV mono-infection, 204/350 (58.3%) individuals have HCV/HIV co-infection while 57/350 (16.9%) were found negative as summarized in Figure 1a and 1b.

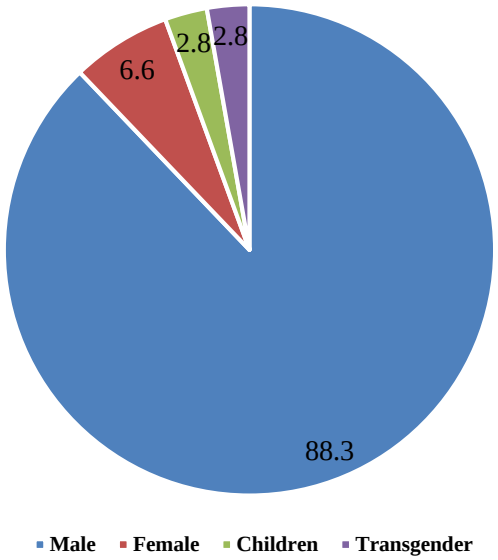

Figure 1a. Demographic Analysis: Gender wise Distribution

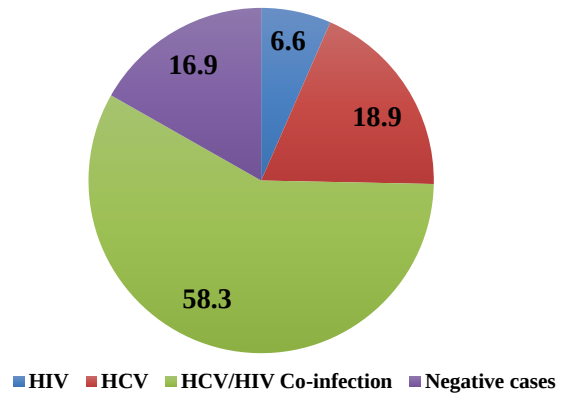

Figure 1b. Demographic Analysis: Prevalence of HCV and HIV Infections

Distribution of HCV genotypes in IDUs

Out of 350 samples, the distribution of HCV genotypes percentage was as follows: 3a accounted

for 179 samples (51.1%), 2a for 12 samples (3.4%), 1a for 25 samples (7.1%), untypeable for 102 samples (29.1%), and mixed genotypes for 32 samples (9.1%). According to bivariate analysis, there is a statistically weak negative r=-0.155 between the age of patients and HCV genotypes (p=0.018). The results are depicted in Figure 2.

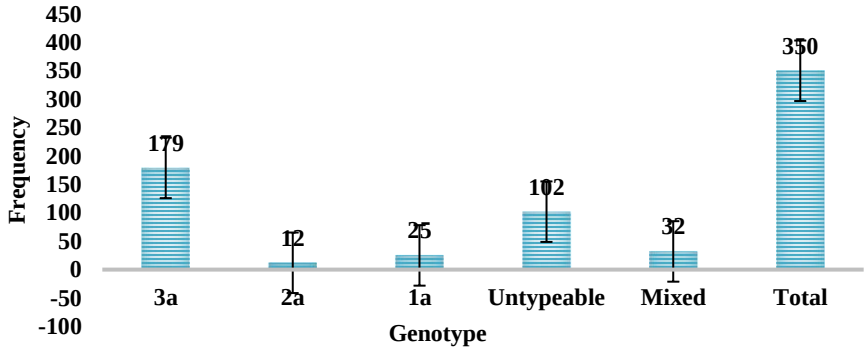

Figure 2. Distribution of HCV genotypes in IDUs

Analysis of viral load with genotypes

Out of 350 cases, 71 (20.3%) had viral load ND, similarly 37 (10.6%) have viral load <1000, 86 (24.6%) have viral load 1001-10000, 106 (30.3%) have viral load in between 10001-100000, however 50 (14.3%) have viral load >100000. According to bivariate analysis, there is a statistically weak negative r=-0.279 between the viral load of patients and HCV genotypes (p=0.000) Figure 4.3.

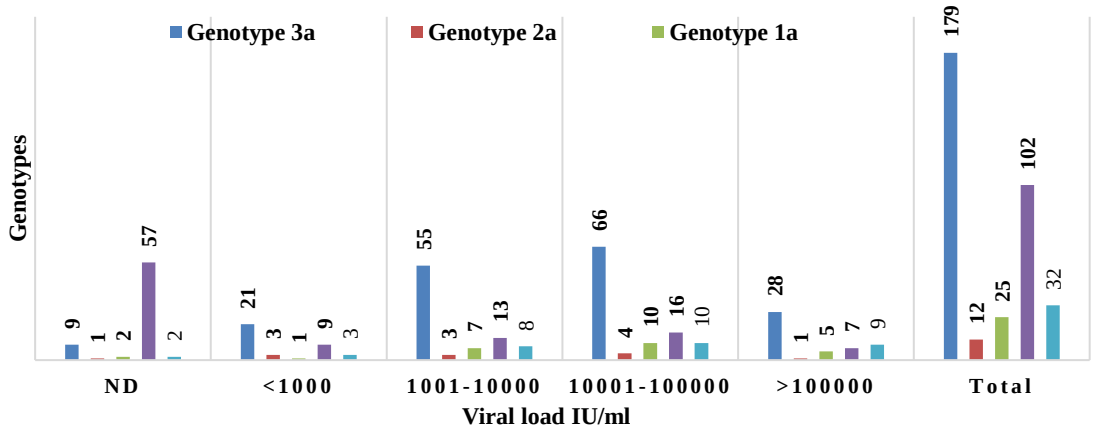

Figure 3. Analysis of viral load with genotypes

Analysis of gender/number of patients with viral Load

The viral load has been divided into different categories, in each category viral load of males was observed higher than females, children, and transgender. According to bivariate analysis, there is a statistically weak negative r=-0.022 between the viral load of patients and the gender of patients (p=0.230) Figure 4.

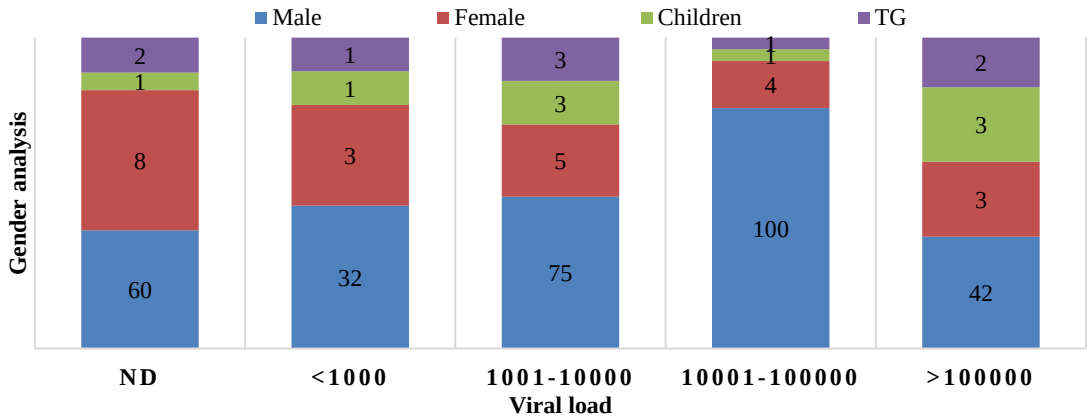

Figure 4. Analysis of gender/number of patients with viral load

Number of HCV/HIV Co-infection with viral load

Out of 204 (58.3%) IDUs having HCV/HIV co-infection only, ND was 05 (1.4%), 19 (5.4%) have viral load <1000, 56 (16.0%) have viral load range from 1001-10000, 83 (23.7%) have viral load range from 10001-100000 and 41 (11.7%) have >100000 viral loads. According to bivariate analysis, there is statistically moderate positive r=0.522 between the viral load of patients and HCV/HIV infection (p=0.000) Figure 5.

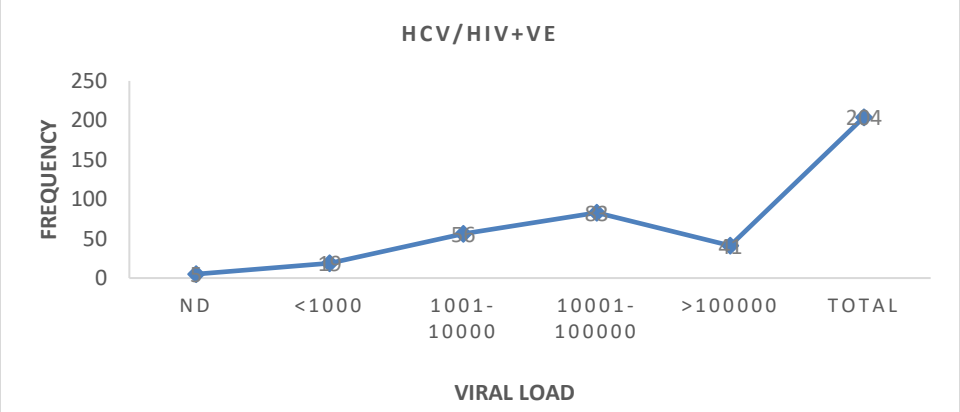

Figure 5. Number of HCV/HIV Co-infection with viral load

DISCUSSION

The current study aimed to determine the diagnostic performance of HCV/HIV co-infection in IDUs through rapid antigen tests and RT-PCR for particular symptomatic individuals and evaluate the genotype of HCV to reduce the severity of HCV/HIV co-infection and other AIDS-related complications. The total number of symptomatic individuals in the current study was 350; primary screening was done by ICT and then compared to RT-PCR. Among total (n=350) IDUs, 66 (18.9%) individuals tested positive for HCV mono-infection, 23 (6.6%) were found HIV positive, and 204 (58.3%) individuals tested positive for HCV/HIV co-infection. Males were greater in number such as 309 (88.3%), in which 191 (54.6%) were HCV/HIV co-infected, 14 (4.0%) HIV positive, and 53 (15.2%) were found HCV positive. Similarly, the number of females was 23 (6.6%) among them 08 (2.3%) were HCV/HIV co-infected. Children were 09 (2.6%) of which 02 (0.6%) were co-infected, while among 09 (2.6%) transgender 03 (0.9%) were HCV/HIV co-infected. Our findings are consistent with the study (Zahra et al., 2022)in which the frequency of HCV/HIV co-infection was high at 98.77%. In our study, the patients were categorized into four age groups <20, 21-40, 41-60, and 61-80 years. The average age of patients was 34 years. The highest prevalence of HCV/HIV co-infection was observed in the age group 21-40 (33.1%), followed by 41-60 (16.9%), <20 (6.6%), 61-80 (1.7%). In contrast to a previous study (Akhtar et al., 2022) the highest prevalence was observed in patients age > 40 years.

The current study also evaluated HCV genotypes in IDUs having HCV/HIV co-infection and HCV mono-infection. In our study out of 350 cases, the most prevalent genotype was 3a genotype, 179 (51.1%), followed by 2a genotype, 12 (3.4%), 1a genotype, 25 (7.1%), untypeable genotype, 102 (29.1%) and mixed genotype, 32 (9.1%). Our study was consistent with the previous study conducted by Solomon *et al* (Solomon et al., 2019), in which the most prevalent genotype was genotype 3 (58.1%), subtype 3a (65%) subtype 3b (35%) (Waqas et al., 2015) revealed the frequency of genotype 3a in 227 (60.5%) patients, 3b in 24 (6.4%). In contrast to the research (Grzeszczuk et al., 2015) in which they reported that the most common genotype was 1b was 37.3%, then genotype 3 was 32.1% and genotype 4 as 30.6%. In our study, we also evaluated the viral load of HCV/HIV co-infection in IDUs in five groups such as ND, <1000 copies/ml, 1001-10000 copies/ml, 10001-100000 copies/ml, and >100000 copies/ml. Males had more viral load (12.5%) than females (0.9%), children (0.9%), and TG (0.6%). Our study was consistent with the research (Shafique and Javed, 2022) in which viral load in males was higher than in females and TG. In this study, we also observed higher viral load in patients infected with genotype 3a than with genotype 2a, 1a, untypeable and mixed. Hence, in contrast to the research (Rong et al., 2012) in which viral load in genotype 1 was higher. This study has potential limitations such as sample size, and most patients did not agree to share their consent. Furthermore, 29% of samples had untypeable genotypes and all these patients were HCV-RNA positive and had sufficient viral load. Due to the lack of a sequencing facility in Pakistan, we were unable to sequence untypeable samples and therefore might be genotyped by sequencing method to find the exact genotype.

Conclusion

The current study concludes that in IDUs the frequency of HCV/HIV co-infection is higher than in those who have HCV, HIV mono-infection. Different HCV genotypes are also determined but the most predominant genotype is 3a (51.1%), followed by genotype 2a (3.4%), 1a (7.1%), untypeable (29.1%) and mixed (9.1%). Furthermore majority of the infected people belong to the age

group 21-40 high viral loads are observed in male age groups.

Recommendations

The following are the recommendations:

- Preventive measures should be taken to reduce the transmission of HCV/HIV co-infection among IDU patients by focusing on blood transfusion, using syringes, tattooing, and being a sex worker
- To overcome the burden of these communicable diseases large-scale, multicentre, and multistate studies should be conducted across Pakistan
- To increase therapeutic power, we will need to resolve the immunological defect that is responsible for the declined cellular immune response to HCV in HCV/HIV co-infected individuals.

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Declarations

Declaration of Interest Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

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Author's contributions

KB, S and JU conducted research and wrote initial draft of manuscript. KN, SH, MAK, AWK, SF and A.H collected the literature and wrote the manuscript and edit the manuscript in original. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for Publication

Not applicable