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Hepatitis C Serological Markers Among the Apparently Healthy and Educated Population: Prevalence, Molecular Characterization, and Risk Factors Assessment

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Key words
HCV prevalence, Asymptomatic HCV, HCV genotypes, Anti-HCV antibodies, Prevalence of hepatitis C, Hepatitis C in Azad Kashmir, Risk factors of hepatitis C

ABSTRACT

The prevalence and molecular characteristics of hepatitis C virus (HCV) infection in Azad Jammu and Kashmir, Pakistan, remain poorly documented. To address this gap, a cross-sectional study was conducted among 7,015 apparently healthy students and employees of the University of Azad Jammu and Kashmir, Muzaffarabad. The study aimed to determine the seroprevalence of HCV, identify circulating genotypes, and assess associated risk factors. Initial screening for anti-HCV antibodies was performed using immune-chromatographic test (ICT) devices. HCV RNA detection and viral load quantification were carried out using polymerase chain reaction (PCR), and genotype identification was performed using type-specific primers. Data on potential risk factors were collected through a structured questionnaire. Anti-HCV antibodies were detected in 0.93% of participants, and 26 individuals (0.37%) were confirmed to be HCV RNA-positive. Among these individuals, genotype 3a was the most prevalent (50%), followed by genotype 1a (23.07%), genotype 3b (15.38%), and genotype 2b (11.54%), with a similar predominance of genotype 3a observed in both males and females. Of the 26 isolates, 10 (38.46%) were successfully sequenced for the NS5B gene, revealing the presence of genotypes 2 and 3, with subtype 3a being the most frequent. Phylogenetic analysis showed that most isolates clustered closely with Pakistani reference sequences, while some genotype 3b isolates showed genetic relatedness to strains from China. A higher prevalence of HCV infection was observed among males, and the risk increased with advancing age. Viral loads ranged from 1,754 to 10,521,733 IU/mL, and elevated liver function tests were observed in 27% of RNA-positive individuals. These findings indicate a 0.93% prevalence of HCV among an educated, asymptomatic population that was largely unaware of its infection status and associated risk factors. Despite the relatively low prevalence, the presence of active HCV infection and circulating genotypes highlights the potential for silent transmission within academic communities. This study emphasizes the need for expanded HCV surveillance, routine screening programs, and targeted awareness campaigns within educational institutions across Azad Jammu and Kashmir. Strengthening preventive strategies, improving early diagnosis, and promoting timely linkage to care are essential steps to reduce HCV transmission and support national efforts toward hepatitis C elimination in the region.

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INTRODUCTION

The prevalence of HCV infection exhibits global variation, with higher rates observed in certain regions, countries, and specific subpopulations such as individuals who inject drugs, prisoners, and people living with HIV (Lazarus *et al*., 2020). It is a global public health issue (Kushner and Reau, 2021). According to the World Health Organization, 58 million people worldwide are believed

to have the hepatitis C virus as of 2019 (CDC, 2021), and each year more than 290000 people die from it (Sheikh *et al*., 2019). With symptoms ranging from moderate to severe, including liver cirrhosis and cancer, the virus can cause both acute and chronic hepatitis. Around the world, the chronic hepatitis C is thought to be present in 58 million persons (WHO, 2022) and 177.5 million adult population is infected with the HCV worldwide putting Africa at the top with a 2.9% prevalence (Petruzziello *et al*., 2016). Genotype 1 is the most common genotype of HCV around the world (Messina *et al*., 2015). Unsafe injections, sharing of personal belongings (razors, toothbrushes, etc.), tattoos (body piercing), transfusion, transplantation, sexual transmission, and from mother to child transmission are the most common routes for hepatitis C transmission (CDC, 2020).

Hepatitis C prevalence among students at educational institutions cannot be accurately predicted by previous studies. However, 0.7% HCV antibody prevalence was reported among teenage students at Babcock University, Nigeria without the assessment of responsible risk factors (Omolade and Adeyemi, 2018). Similar to this, the anti-HCV prevalence among undergraduates of Ladoke Akintola University in Ogbomosho, Nigeria was found 0.4% (Jemilohun *et al*., 2014) and the risk factors were not found. Whereas, Ehab *et al*. (2018) reported about Minia city school students, in Egypt that a 0.7% prevalence of hepatitis C antibodies having a significant relationship with blood transfusion, sharing shaving instruments and toothbrushes (p= 0.029, 0.031, 0.002, respectively) was found. However, 4.7% of Egyptian schoolchildren with HCV Ab-seropositive status were found in 2017. The likelihood of developing a disease was shown to be substantially correlated with a history of blood exposure, frequent intravenous injection, past hospitalization, and blood exposure (Naveen *et al*., 2017). 1.4% of University of Benin, Benin City, Nigeria students have antibodies to the hepatitis C virus (Ogefere *et al*., 2016). In contrast, 0.7% of university staff members in So Paulo, Southeast Brazil, had HCV infection. Age (P=0.01), intravenous drug usage (P0.0001), and hemodialysis (P=0.0004) were the risk factors for HCV infection (Oliveira *et al*., 2015). Similar to Saudi Arabia, among students in health colleges, anti-HCV prevalence was 0.07 percent for women and 0.03 percent for men, respectively. Age and rural/urban origin were correlated with anti-HCV positive statistically significantly (P 0.005) (Al-Ajlan, 2011).

Pakistan has the second-highest burden of HCV infection in the world (Sahibzada *et al*., 2022). Print and electronic media are being used by public health agencies to raise awareness about the viral hepatitis (Hamid *et al*., 2004). The prevalence of hepatitis C in Pakistan has remained stable, and there are no indications of a decrease in spite of the availability of generic direct-acting antiviral medications and the subsequent decrease in treatment costs (Mahmud *et al*., 2019). However, in the tehsil, Daggar district Buner, KPK, Pakistan 34% of subjects (14.9% men, 19.2% women) had HCV RNA found in their bodies. The most prevalent genotypes were genotype 3a and the untypable genotype. It was determined that visits to barbershops, dental checkup, and repeated injections were responsible risk factors (Qamar *et al*., 2021).

In the overall adult population of Pakistan, the prevalence of HCV was 4.95% ± 0.53%. A very high frequency (57% 17.7%) was seen among injectable drug users, and 48.67% 1.75% in a group with several transfusions. The 3a HCV genotype was the most common (Waheed *et al*., 2009). While the 2007–2008 national survey placed the HCV prevalence at 4.8% (Qureshi *et al*., 2010). Whereas, the meta-analyses put the overall population's pooled mean HCV prevalence at 6.2%. An unprecedented HCV pandemic is raging in Pakistan, where one in every 20 people has the disease (Al-Kanaani *et al*., 2018).

The development of a national-level hepatitis C control policy specifically for educational establishments is not feasible. The existing research studies do not provide a comprehensive representation of the overall prevalence of hepatitis C infection in educational institutes in Pakistan. Muzaffarabad, the capital city of the state of AJandK, is home to the University of Azad Jammu and Kashmir Muzaffarabad, which is the oldest university in the state established in 1980. This university attracts individuals from various tribes and districts of AJ and K as well as from different parts of Pakistan. The aim of this study was to assess the prevalence of hepatitis C virus infection, investigate potential responsible factors of the infection spread, and identify the predominant genotypes among the healthy individuals enrolled at the University of AJandK, Muzaffarabad, representing diverse tribal and district backgrounds within the state.

MATERIALS AND METHODS

Inclusion criteria and consent to participate

All the employees (faculty members, administration staff, security staff and non-teaching staff) and students (BS to PhD) above 18 years of age of the University of AJK, Muzaffarabad belonging to both sexes, different ages, departments, ethnic groups, geographical areas of origin, on the base of willingness to participate were included in the study. Written consent with signature was also taken from all the individuals before enrolling them in the study.

Data collection

A questionnaire was used to get information about the age, ethnicity, sex, medical history, surgical history, and geographical area of the participating individuals and the risk factor assessment. In addition to screening, participants were asked to report additional viral hepatitis symptoms such as abdominal discomfort, anorexia, fever, dyspepsia, vomiting, nausea, and jaundice on a pre-structured questionnaire. Screening of each department was conducted on specific days. All departments of UAJK participated in screening.

Sample collection and screening of HCV antibodies

To conduct the initial screening, venipuncture was performed using 5 ml syringes to collect blood samples. Serum was separated from all samples by centrifugation. All the individuals were screened for qualitative anti-HCV antibodies through "Accurate Rapid Anti-HCV Immuno-Chromatographic Test Devices" of Healgen Scientific LLC, Houston, USA. These ICT devices were selected for initial screening because they are rapid, cost-effective, and suitable for large-scale population-based studies, allowing early identification of suspected HCV exposure.

Detection of HCV RNA by PCR

Viral RNA from individuals who tested positive for anti-HCV antibodies was isolated using the optimized TRIzol method, as described by Rauf *et al*. (2013), and subsequently amplified following the protocol of Casanova *et al*. (2014).

Viral load calculation

The quantitative detection of HCV in the serum of infected individuals was performed using real-time PCR. Isolated HCV RNA was amplified through real-time amplification. The quantitative detection of HCV RNA was accomplished using a fluorescent reporter dye probe specific for HCV (Cy3) or HCV IC (FAM) in the Smart Cycler system. For quantification purposes, quantitation standards ranging from 125 to 12,500,000 were utilized.

HCV genotyping and phylogenetic analysis

To determine the specific genotype and subtype of HCV in the PCR-positive samples, cDNA of the HCV core gene was generated using type-specific primers designed based on the HCV core gene sequences available in public databases, following the method described by Nazir *et al*. (2017). The HCV NS5B gene was amplified from 26 isolates, of which 10 (38.46%) were successfully sequenced and analyzed using NCBI tools and MEGA X software.

Liver function tests

Liver function tests (LFTs) were performed on individuals who tested positive for the Hepatitis C virus (HCV) using a Microlab-300 (Merck analyzer). The objective of these tests was to assess the levels of alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin in the participants' blood samples.

Statistical analysis

Data analysis in the study was conducted with SPSS (Version 24.0). Chi-square test and Fisher's exact test were used to determine the differences in prevalence of HCV between different categories of factors.

RESULTS

Overall HCV prevalence

In the initial screening using ICT, 65 individuals (0.93%) tested positive for anti-HCV antibodies. When these 65 samples were further analyzed by PCR, 26 were confirmed positive for HCV RNA, yielding an overall HCV RNA prevalence of 0.37% in the study population (Table I).

Table I. Gender and age-based prevalence of hepatitis C virus (HCV) among the UAJK population (n= 7015).

Category	Subgroup	Number of participants (%)	ICT-based Anti-HCV positive (%)	HCV RNA positive (%)
Overall	Total population	7,015 (100)	65 (0.92)	26 (0.37)
Gender	Male	3,210 (45.75)	43 (1.34)	17 (0.53)
	Female	3,805 (54.24)	22 (0.58)	9 (0.24)
Age group (years)	16–30	6,270 (89.38)	41 (0.65)	10 (0.16)
	31–45	504 (7.18)	12 (2.38)	8 (1.59)
	≥46	241 (3.43)	12 (4.98)	8 (3.32)

Gender based HCV prevalence

Out of the 7,015 participants, 3,210 (45.7%) were male and 3,805 (54.3%) were female. Among them, 43 males (1.34%) and 22 females (0.58%) tested positive for anti-HCV antibodies in the initial ICT screening. The Chi-square test showed a significant association between gender and HCV prevalence (P= 0.000), with a higher frequency of ICT-positive cases among males. Further PCR testing of these ICT-positive samples confirmed HCV RNA in 17 males (0.53%) and 9 females (0.24%). The prevalence of active HCV infection was significantly higher in males compared to females (P = 0.000) (Table I).

Age based HCV prevalence

The mean age of the study population was 23.7 ± 7.2 years. The prevalence of anti-HCV antibodies was 0.65% in the 18–30 years age group, 2.38% in the 31–45 year group, and 4.98% among individuals aged 46–60 years (Table I). A significant increase in HCV prevalence was observed with advancing age (P= 0.000), indicating a direct association between age and HCV infection.

HCV prevalence and participant's category

The study participants were categorized into two groups: students and employees. A total of 5,954 students and 1,061 employees were enrolled. Among them, 16 students (0.27%) and 11 employees (1.0%) tested positive for anti-HCV antibodies. HCV RNA was detected in 16 students (0.27%) and 10 employees (0.93%). The prevalence of HCV was significantly higher among employees compared to students (P = 0.000) (Supplementary Table I).

HCV prevalence among various tribes

The study population belonged to twelve tribes from Azad Jammu and Kashmir, the Northern Areas, and other regions of Pakistan (Table II). The highest prevalence of hepatitis C was observed in the Balti tribe (1%). The prevalence in other tribes was as follows: Pakhtoon (0.86%), Qureshi (0.82%), Syed (0.57%), Sheikh (0.55%), Awan (0.51%), Rajpoot (0.38%), Sudhan (0.32%), Khawaja (0.22%), Abbasi (0%), Chaudhary (0%), and Mughal (0%).

HCV prevalence based on geographical belongings of participants

The studied participants belonged to 13 different geographical areas included 10 districts of AJK and 3 regions of Pakistan (Table II). Maximum participants belonged to district Muzaffarabad (3913) and minimum to district Bhimber (50). Highest prevalence of HCV was found in the participants belonging to district Sudhnoti (1.41%), which was followed by the participants from following regions in descending order: Northern Areas (1.0%), Punjab (0.79%), KPK (0.78%), Hattian (0.65%), Neelum (0.53%), Poonch (0.30%), Muzaffarabad (0.25%), Bagh (0.17%), Bhimber (0%), Haveli (0%), Kotli (0%), and Mirpur (0.0%).

HCV viral load among participants

Number of copies of HCV RNA per milliliter of the sample was detected for HCV RNA-positive individuals through real-time PCR. Viral load varied among individuals, it was as low as 1,754 IU/ml and as high as 36,79,145 IU/ml. On the base of viral load, individuals were divided into three groups. Among the 26 positive participants, 9 had low viral load, ranging from 1754 IU/ml to 50,000 IU/ml, 5 had intermediate levels of viral load ranging from >50,000-1,00,000 IU/ml while 12 had a viral load higher than1,00,000 IU/ml (Supplementary Table II).

Table II. Ethnicity and geography-based prevalence of hepatitis C virus (HCV) among the studied population.

Category / Subgroup	Number of participants (%)	ICT anti-HCV positive (%)	HCV RNA positive (%)
Ethnicity			
Abbasi	421 (6.00)	1 (0.23)	0 (0.00)
Awan	788 (11.23)	10 (1.27)	4 (0.51)
Balti	300 (4.27)	10 (3.33)	3 (1.00)
Chaudhary	568 (8.10)	1 (0.17)	0 (0.00)
Khawaja	886 (12.63)	9 (1.01)	2 (0.22)
Mughal	638 (9.09)	1 (0.16)	0 (0.00)
Pakhtoon	233 (3.32)	12 (5.15)	2 (0.86)
Qureshi	245 (3.49)	2 (0.82)	2 (0.82)
Rajpoot	1,572(22.40)	8 (0.51)	6 (0.38)
Sheikh	183 (2.60)	1 (0.55)	1 (0.55)
Sudhan	310 (4.42)	2 (0.64)	1 (0.32)
Syed	871 (12.42)	8 (0.92)	5 (0.57)
Geography			
Bagh	569 (8.11)	2 (0.35)	1 (0.17)
Bhimber	50 (0.71)	1 (2.00)	0 (0.00)
Hattian	459 (6.54)	6 (1.30)	3 (0.65)
Haveli	144 (2.05)	1 (0.69)	0 (0.00)
KPK	382 (5.44)	10 (2.62)	3 (0.78)
Kotli	145 (2.06)	1 (0.69)	0 (0.00)
Mirpur	73 (1.04)	0 (0.00)	0 (0.00)
Muzaffarabad	3,913(55.78)	20 (0.51)	10 (0.25)
Neelum	379 (5.40)	5 (1.32)	2 (0.53)
Northern Areas	300 (4.27)	10 (3.33)	3 (1.00)
Poonch	332 (4.73)	2 (0.60)	1 (0.30)
Punjab	127 (1.81)	5 (3.94)	1 (0.79)
Sudhnoti	142 (2.02)	2 (1.41)	2 (1.41)

KPK, Khyber Pakhtunkhwa

Liver function test

Liver function tests (LFTs) were conducted on HCV positive individuals to trace the active infection. Out of the 26 positive participants, 19 (73%) had all three LFT markers within the normal range while 7 (27%) had elevated levels of Bilirubin, ALT, and ALP. This elevation in LFT suggests liver damage up to some extent in these individuals, likely attributed to the active HCV infection (Supplementary Table III).

Table III. Identification results of HCV subtypes based on basic alignment search tools.

Code	Our Seq. accession number	Species	Max score	Total score	Query coverage	E. value	Similarity	Accession length	Accession number
UAJK-01-PK	ON351045	HCV	534	534	100%	2e-147	97.74%	313	MW234406.1
UAJK-02-PK	ON351046	HCV	512	512	100%	9e-141	96.74%	313	MW234405.1
UAJK-03-PK	ON351047	HCV	523	523	100%	4e-144	97.10%	313	MW234404.1
UAJK-04-PK	ON351048	HCV	497	497	100%	2e-136	96.07%	308	MW234403.1
UAJK-05-UAJK	ON351049	HCV	494	494	100%	3e-135	96.33%	314	MW234402.1
UAJK-06-UAJK	ON351050	HCV	520	520	100%	5e-143	96.50%	314	MW234401.1
UAJK-07-UAJK	ON351051	HCV	464	464	100%	2e-126	95.52%	313	MW234400.1
UAJK-08-UAJK	ON351052	HCV	647	647	100%	0.0	95.12%	421	MW016379.1
UAJK-09-UAJK	ON351053	HCV	545	545	100%	1e-150	94.37%	355	MT210568.1
UAJK-10-UAJK	ON351054	HCV	392	392	100%	1e-104	93.23%	272	KY628329.1

HCV genotyping and phylogenetic analysis

Of the 26 HCV RNA-positive participants, the most prevalent genotype found was 3a (50%) while 6 of the participants were found with genotype 1a (23.07%), 3 with genotype 2b (11.54%), and 4 (15.38%) with genotype 3b. Most prevalent genotype in males was 3a (47.05%), followed by 1a (23.52%), 2b (17.65%) and 3b (11.76%) respectively. In females, the most prevalent genotype was also 3a (55.55%), whereas 1a and 3b had the same prevalence (22.22%) and no female had 2b genotype (Supplementary Table IV).

The HCV NS5B gene of 26 isolates was amplified, of which 10 (38.46%) were successfully sequenced and analyzed (Table III). From these 10 sequenced isolates, HCV genotypes were determined, and phylogenetic analysis was performed. Genotypes 2 and 3 were detected in these samples. Among the genotype 3 isolates, five (5/10, 50%) belonged to subtype 3a, and two (2/10, 20%) belonged to subtype 3b. Three isolates (3/10, 30%) were identified as genotype 2a. Subtype 3a was the most predominant in this region, while subtype 2a was less frequent.

A phylogenetic tree was constructed by comparing the HCV isolates with reference sequences from different countries obtained from NCBI. HCV isolate ON351053.1 showed 93% identity with reference sequence MT210568.1 from Pakistan, confirming it as subtype 3a with a bootstrap value of 65%. Similarly, isolate ON351051.1 exhibited 95% identity with reference MW234400.1 from Pakistan, classified as subtype 3a with a 96% bootstrap value. Isolate ON351046.1 displayed 96% identity with reference MW234405.1, supported by a high bootstrap value of 98%. Isolates ON351046.1 and ON351054.1 showed 96% and 93% identity, respectively, with Pakistani genotype 3a sequences MW234405.1 and KY628329.1. In the phylogenetic tree (Fig. 1), these sequences did not form a separate cluster, suggesting that all these isolates are likely subtype 3a of Pakistani origin. The phylogenetic analysis of HCV NS5B genotype 3 isolates confirmed their genotype and subtype identities as indicated by BLAST and the HCV database.

Isolate ON351048.1 displayed a high bootstrap value (81%) with subtype 3b reference MW234403.1 from Pakistan, and BLAST analysis showed 96% identity with the same reference sequence. Isolate ON351052.1 showed 95% identity with genotype 3b Pakistani sequence MW016379.1, clustering in the phylogenetic tree with a 76% bootstrap value (Fig. 2). These isolates also demonstrated close genetic links with sequences from China (KF292168.1, KT735832.1, KM285058.1, and KM285057.1) and Pakistan (MW016378.1), suggesting that the Pakistani isolates may have originated from China. In the phylogenetic tree, isolate ON351052.1 (subtype 3b) appeared at the base along with corresponding reference sequences.

Among genotype 2 isolates, ON351049.1 and ON351047.1 showed 96% and 97% identity, respectively, with Pakistani subgenotype 2b sequences MW234402.1 and MW234404.1. These isolates clustered together in the phylogenetic tree with a maximum bootstrap value greater than 75%. Another isolate, ON351050.1 (subtype 2b), was closely related to Pakistani sequence MW234401.1, forming a cluster with a bootstrap value of 98% (Fig. 3). The analysis confirmed that all three genotype 2b isolates originated from Pakistan.

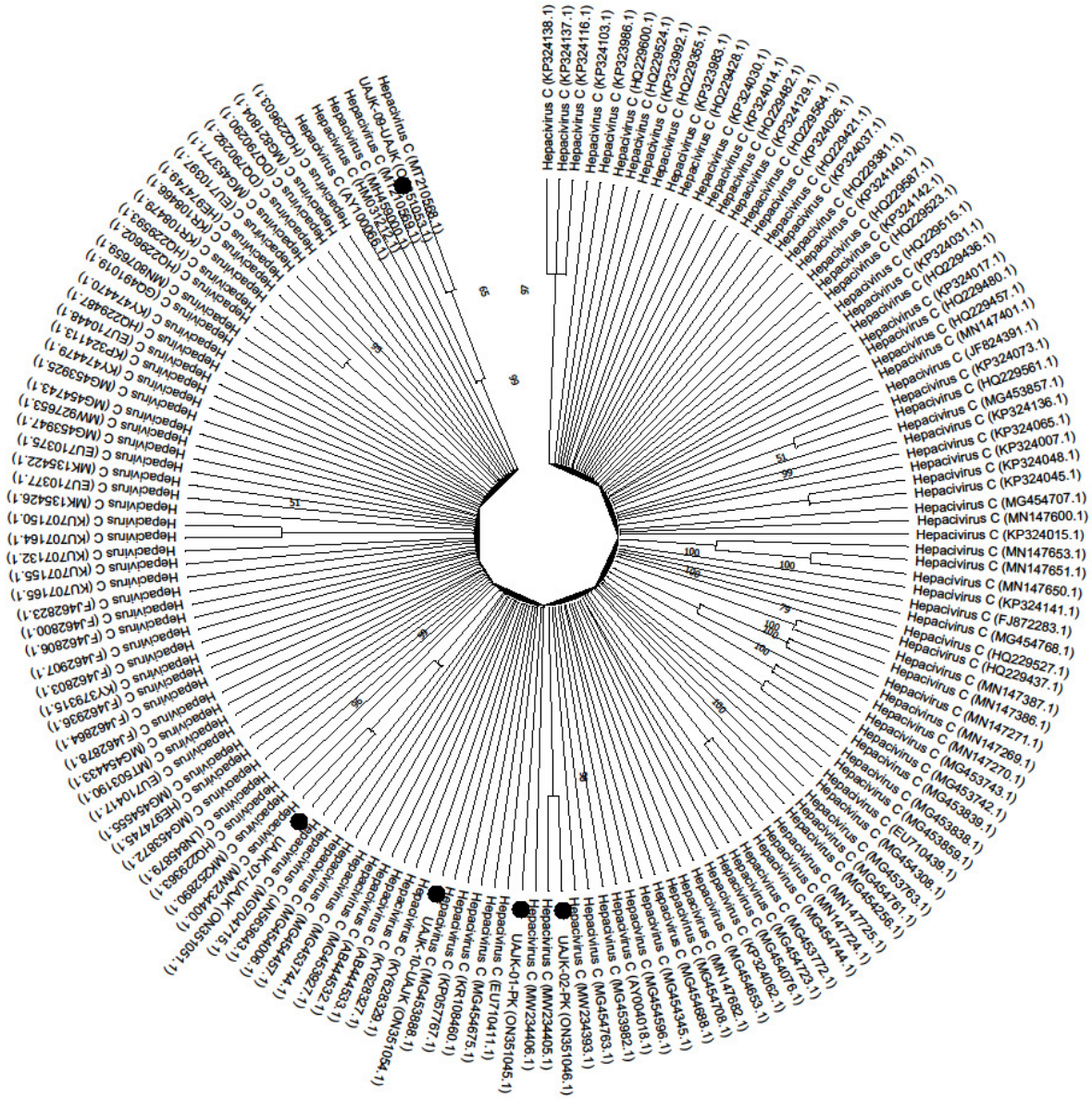

Fig. 1. Phylogeny of HCV NS5B genes in HCV patients. Tree was constructed using Neighbor-Joining method with maximum composite likelihood model. Study sequences are marked with black blocks showing genotypes 3a.

Risk factors assessment

Association of various risk factors was calculated with HCV diagnosis in the study. Significantly higher (*P<0.001*) percentage of HCV positive study participants was found in those participants having history of blood transfusion compared to those having no history (1.71% vs. 0.1%). The participants having history of any surgery also had a higher percentage of HCV positive individuals (*P<0.001*) compared to those having no history of surgery (5.13% vs. 0.32%). Similarly, the participants with tattooing or piercing on their bodies had significantly higher infection percentage (*P=0.001*) compared to those having no tattooing or piercing (0.72% vs. 0.16%). So, the factors found to be significantly associated with detection of HCV infection in the study were: transfusion history, history of any surgery, and tattooing/piercing while no association of HCV infection was found with jaundice history, dental procedure, any immune suppressant history, hospitalization history, and transplantation (Table IV).

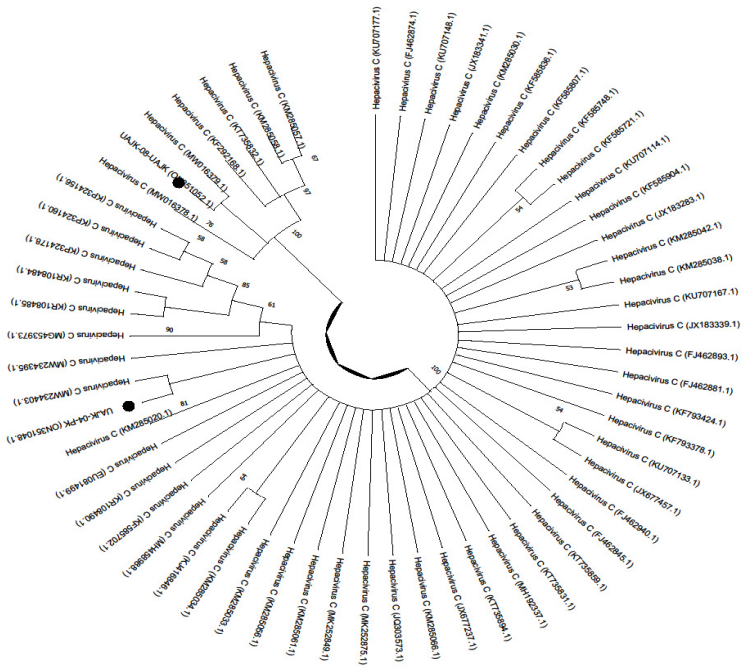

Fig. 2. Phylogeny of HCV NS5B genes in HCV patients. Tree was constructed using Neighbor-Joining method with maximum composite likelihood model. Study sequences are marked with black blocks showing genotypes 3b.

Table IV. Risk factor assessment regarding HCV transmission among the participants.

Assessed factors	Responses	Overall responses	Frequencies diseased (anti-HCV antibody positive)	Frequencies non-diseased	Two tailed Chi-square p < (at 0.05)
Jaundice/ hepatitis history of self or in family	Yes	100(1.4%)	0	100	0.3300
	No	6915(98.6%)	65	6850	
Blood transfusion	Yes	1170(16.7%)	27	1143	<0.0001*
	No	5845(83.3%)	38	5807	
Underwent any other surgery	Yes	3309(47.2%)	01	3308	<0.0001*
	No	3706(52.8%)	64	3642	
Any immunosuppressive disease	Yes	656(9.4%)	01	655	0.0297
	No	6359(90.6%)	64	6295	
Tattooing or piercing	Yes	4381(62.5%)	24	4357	<0.0001*
	No	2634(37.5%)	41	2593	
Underwent dental surgery	Yes	2630(37.5%)	26	2604	0.6746
	No	4385(62.5%)	39	4346	
Exposure to injections	Yes	7015(100%)	65	6950	N.A
	No	0(0%)	0	0	
Sharing of personal belongings	Yes	7015(100%)	65	6950	N.A
	No	0(0%)	0	0	
Visit to beauty parlor/ Barber shop	Yes	7015(100%)	65	6950	N.A
	No	0(0%)	0	0	
More than one time Hospitalized	Yes	726(10.3%)	07	719	0.9111
	No	6289(89.7%)	58	6231	
Transplantation	Yes	02(0.03%)	0	2	0.8912
	No	7013(99.97%)	65	6948	

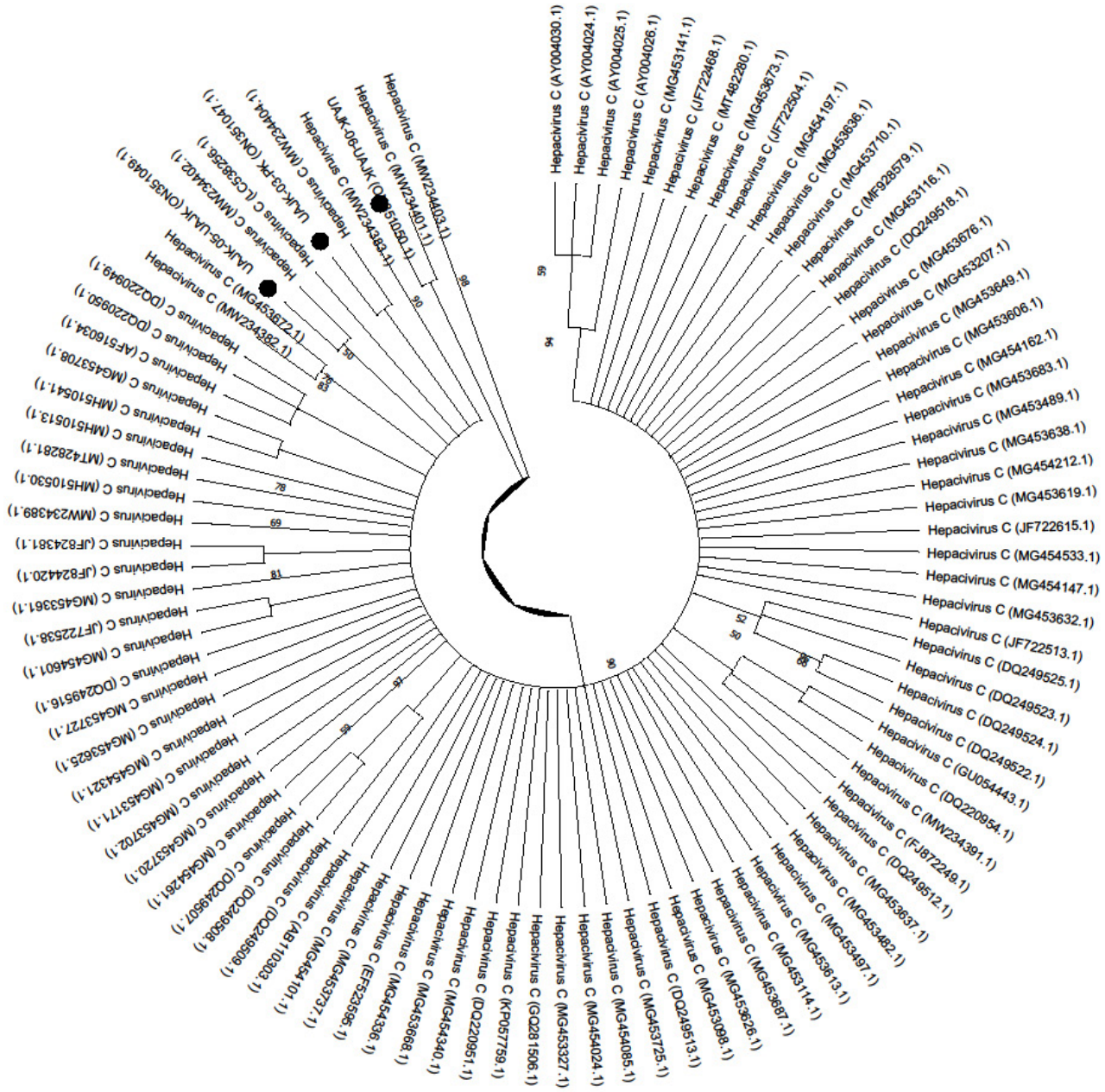

Fig. 3. Phylogeny of HCV NS5B genes in HCV patients. Tree was constructed using Neighbor-Joining method with maximum composite likelihood model. Study sequences are marked with black blocks showing genotypes 2b.

DISCUSSION

The present study was conducted among a healthy population comprising students, teachers, and employees of Azad Jammu and Kashmir University, Muzaffarabad, to estimate the true prevalence of hepatitis C virus (HCV) antibodies and HCV RNA and to identify associated risk factors. A total of 7,015 students and employees were enrolled in the study. None of the participants was aware of their HCV status, nor had any been previously diagnosed as HCV-positive. Moreover, no participant exhibited symptoms suggestive of active HCV infection. HCV antibodies were detected in 0.93% of the participants, whereas HCV RNA was identified in 0.37% (n= 26) of the total study population. Genotype 3a was the most prevalent genotype, accounting for 50% of the HCV-

positive cases. Overall, 26 participants (0.37%) were confirmed to be HCV RNA-positive. A higher prevalence of HCV infection was observed among males compared to females, and HCV prevalence increased with advancing age.

Ehab *et al*. (2018) reported about Minia city school students, in Egypt that a 0.7% prevalence of hepatitis C antibodies was found with a significant relationship with blood transfusion, sharing shaving instruments and toothbrushes (p= 0.029, 0.031, 0.002, respectively). However, 4.7% of Egyptian school children were diagnosed anti-HCV seropositive. Frequent IV-injection, history of hospitalization, blood transfusion, and blood exposure was found associated with disease prevalence (Neveen *et al*., 2017).

In contrast, 65 individuals (0.93%) in the present study tested positive for anti-HCV antibodies, including 43 males (1.34%) and 22 females (0.58%). A statistically significant association was observed between hepatitis C infection and a history of blood transfusion (p= 0.0001), surgical procedures (p= 0.0001), the presence of any immunosuppressive disease (p= 0.0297), and tattooing or body piercing (p= 0.0001). These findings are consistent with previous studies and align with the World Health Organization's recognition of blood transfusion as a major risk factor for hepatitis C transmission. Niu *et al*. (2016) also reported similar results, showing that blood transfusion was a statistically significant risk factor for HCV infection in China as well as in other countries, including the United States. Collectively, these findings highlight the importance of identifying and addressing key risk factors contributing to the spread of HCV.

At the University of Benin, Nigeria, the prevalence of hepatitis C was reported to be 1.4%, with smoking, skin piercing, and alcohol consumption identified as associated risk factors (Ogefere *et al*., 2016). In contrast, a study conducted among university employees in Southeastern Brazil reported an HCV prevalence of 0.7%, with age ($p = 0.01$), intravenous drug use ($p < 0.0001$), and hemodialysis ($p = 0.0004$) identified as significant risk factors (Oliveira *et al*., 2015). The prevalence of hepatitis C reported by Ogefere *et al*. (2016) in a Nigerian university setting (1.4%) is higher than the prevalence observed in the present study (0.93%). This difference may be partly explained by variations in sample size and study population. Ogefere *et al*. (2016) conducted their study among 500 participants and identified alcohol consumption, smoking, and skin piercing as major risk factors. In contrast, the present study included a larger population of 7,015 participants and identified blood transfusion ($p = 0.0001$), surgical procedures ($p = 0.0001$), the presence of immunosuppressive diseases ($p = 0.0297$), and tattooing or body piercing ($p = 0.0001$) as significant risk factors associated with hepatitis C infection.

In Wuhan, Central China, the prevalence of hepatitis C was evaluated by age and gender. Niu *et al*. (2016) reported that before the age of 60 years, female patients had a higher prevalence of HCV infection than males; however, this trend reversed after the age of 60. In the present study, the overall gender-based prevalence of hepatitis C was 1.34% among males and 0.58% among females. Unlike the findings of Niu *et al*. (2016) , HCV prevalence in the present study was higher among males than females across all age groups. Similarly, Ayano *et al*. (2018) reported a higher prevalence of HCV infection in men compared to women (9.16% vs. 5.43%), with a statistically significant association between gender and hepatitis C prevalence. Consistent with these findings, the present study also demonstrated a higher prevalence of hepatitis C among men than women.

Niu *et al*. (2016) reported that the predominant HCV genotype was genotype 1 (73.39%), followed by genotype 2 (17.14%), genotype 3 (5.25%), and genotype 6 (3.22%). Within genotype 1, subtype 1b was the most prevalent, accounting for 71.98% of cases, while subtype 2a represented 17.14%. In contrast, the distribution of HCV genotypes in the present study differed. Among the HCV-positive participants, 23.07% were infected with genotype 1a, 11.54% with genotype 2b, 50% with genotype 3a, and 15.38% with genotype 3b. Previous studies have reported the rare occurrence of genotype 5a (Ahmad *et al*., 2010; Afridi *et al*., 2014; Ayano *et al*., 2018). In Pakistan, genotype 3a has been reported as the most common genotype, accounting for 40.96% of cases, followed by genotypes 3b and 1a.

In Saudi Arabia, genotype 4 has been reported as the most prevalent HCV genotype, followed by genotypes 1b and 1a (Attaullah *et al*., 2011; Al-Ashgar *et al*., 2013). In contrast, studies conducted in China, Jordan, Iran, Turkey, and Israel have reported genotype 1 as the predominant genotype, with prevalence ranging from 56.2% to over 70% (Shemer-Avni *et al*., 1998; Bdour, 2002; Bozdayi *et al*., 2004; Idrees *et al*., 2009; Bokharaei-Salim *et al*., 2013; Jahanbakhsh *et al*., 2013). In Pakistan, Haqqi *et al*. (2019) reported genotype 3a as the most prevalent HCV genotype, along with the presence of genotypes 3b (9.05%), 2a (6.70%), 1a (6.22%), and 1b (2.39%). Similarly, Kazmi *et al*. (2022) identified genotype 3a as the predominant genotype (46%) among Kashmiri participants, followed by genotypes 2a (30%) and 1a (24%). Collectively, these findings highlight the consistently high prevalence of genotype 3a and its subtypes within the studied populations.

Among the Saora, Lodha, Khadia, Juanga, and

Mankidia tribes of India, the prevalence of hepatitis C was reported as 0%, 3.3%, 5.7%, 8.5%, and 13.4%, respectively, with HCV genotype 1b identified as the most common strain. Shared razor use, shaving by barbers, tattooing, and repeated injections were identified as significant risk factors (Kar *et al*., 2019). In contrast, the present study observed the highest HCV prevalence among the Pakhtoon tribe (5.15%), followed by the Balti (3.33%), Awan (1.27%), Khawaja (1.01%), Syed (0.92%), Qureshi (0.82%), Sudhan (0.64%), Sheikh (0.55%), Rajpoot (0.51%), Abbasi (0.23%), Chaudhary (0.17%), and Mughal (0.16%) tribes of Azad Jammu and Kashmir, Pakistan. In the present study, HCV genotype 1a was the most prevalent (23.07%). Blood transfusion ($p = 0.0001$), surgical procedures ($p = 0.0001$), the presence of immunosuppressive diseases ($p = 0.0297$), and tattooing or body piercing ($p = 0.0001$) were identified as significant risk factors associated with hepatitis C infection.

In the general population of Azad Kashmir, 7.5% of individuals tested positive for HCV antibodies. Although genotypes 2a, 1b, 3b, and an unclassified strain were detected, genotype 3a was the predominant genotype (Rauf *et al*., 2013). Similarly, a study conducted at the District Headquarters Hospital in Kotli, Azad Kashmir, reported an HCV prevalence of 6.38%. Blood transfusion was identified as the most common risk factor (34.36%), followed by dental surgery (24.54%) (Saleem *et al*., 2011).

Mahmud *et al*. (2019) conducted a survey to estimate the mean prevalence of HCV in the general population across several regions of Pakistan. Sindh reported the highest prevalence (7.0%), followed by the Islamabad Capital Territory (6.9%), Khyber Pakhtunkhwa (6.6%), Azad Jammu and Kashmir (5.8%), Balochistan (5.8%), Punjab (5.6%), and the former Federally Administered Tribal Areas (0.9%). Both previous studies and the present investigation demonstrate that the prevalence of HCV varies across different regions of Pakistan.

In the present study, participants from Punjab showed the highest prevalence of HCV infection (3.93%), followed by the Northern Areas (3.33%), Khyber Pakhtunkhwa (2.62%), Bhimber (2.00%), Sudhnoti (1.41%), Neelum (1.31%), Hattian (1.30%), Haveli (0.69%), Kotli (0.69%), Poonch (0.60%), Muzaffarabad (0.51%), and Bagh (0.35%). No HCV-positive cases were detected among participants from Mirpur. In response to the burden of hepatitis, several provincial-level initiatives have been implemented. Notably, the Punjab Hepatitis Act 2018 represents a significant policy measure aimed at strengthening hepatitis control within the province (The Punjab Hepatitis Act, 2018). Similarly, in Azad Jammu and Kashmir, the Hepatitis Control Program operates under the Government of AJ and K, Pakistan, with the objective of reducing the burden of viral hepatitis.

Kalita *et al*. (2021) investigated the phylogeny and genetic homology of hepatitis C virus (HCV) strains at a dialysis unit in northeast India. The study included 196 participants, and phylogenetic analysis revealed that 29 sequences clustered within genotype 3, with subtype 3f being observed. High sequence similarities were detected among the isolated sequences, ranging from 75% to 100% homology. Similarly, Hussain and Idrees (2013) examined the evolutionary and molecular phylogenetic relationships of HCV strains belonging to genotype 1a. Their results indicated that the studied HCV strain was genetically closer to a German strain (0.06557 nucleotide divergence; 0.139449 amino acid divergence) and more distant from a Danish strain (0.29400 nucleotide divergence; 0.819646 amino acid divergence).

However, Mumtaz *et al*. (2020) conducted a study in Peshawar to analyze the genetic diversity of HCV genotype 3a based on the complete core protein. Phylogenetic analysis revealed that most HCV 3a strains in their study were genetically closer to previously reported HCV 3a strains from Pakistan and India. In the present study, seven sequenced isolates belonged to genotype 3, of which five were subtype 3a (accession numbers ON351045.1, ON351046.1, ON351051.1, ON351053.1, and ON351054.1). These isolates were genetically closer to HCV 3a strains from Pakistan. Two isolates, ON351048.1 and ON351052.1, belonged to subtype 3b and showed high bootstrap values when compared with reference 3b sequences from Pakistan. These isolates also clustered closely with sequences from China, suggesting a possible Chinese origin of these Pakistani isolates. The remaining three successfully sequenced isolates belonged to genotype 2, subtype 2b. Isolates ON351049.1 and ON351047.1 showed 96% and 97% sequence identity, respectively, with Pakistani 2b reference sequences. The third isolate, ON351050.1, also of subtype 2b, was closely related to Pakistani sequences with a maximum bootstrap support of 98% in the phylogenetic tree. Overall, the origin of all three genotype 2b isolates was determined to be Pakistani.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates a 0.93% prevalence of hepatitis C virus infection among an educated and apparently healthy population who were largely unaware of their infection status and associated risk factors. The findings highlight the presence of undiagnosed HCV infection even within low-risk, educated groups, emphasizing the silent nature of the disease. There is a

clear need for expanded epidemiological studies targeting apparently healthy populations, particularly within educational institutions across Azad Jammu and Kashmir, to determine the true burden of hepatitis C in the region. Routine screening programs, awareness campaigns, and early diagnostic strategies should be strengthened to improve timely detection and reduce transmission. The results of this study may assist health authorities in developing evidence-based screening policies and targeted preventive strategies, which are essential for controlling the continued spread of hepatitis C not only in AJandK but also at the national level in Pakistan.

Study limitations

This study has some limitations that should be considered when interpreting the findings. First, the study population was limited to students and employees of a single university, which may not fully represent the general population of Azad Jammu and Kashmir. Therefore, the results cannot be generalized to all demographic or socioeconomic groups in the region. Second, the assessment of risk factors was based on self-reported questionnaire data, which may be subject to recall bias or underreporting, particularly for sensitive exposures such as medical history or behavioral practices. Third, although HCV RNA-positive samples were identified, sequencing was successfully performed for only a subset of isolates, which limited the depth of molecular and phylogenetic analysis. In addition, the cross-sectional study design does not allow for assessment of temporal relationships or causality between risk factors and HCV infection. Despite these limitations, the study provides important baseline data on HCV prevalence, genotypic distribution, and molecular characteristics among an apparently healthy and educated population in AJ and K.

DECLARATIONS

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IRB approval

The research was approved by the Board of Advanced Studies and Research (BASR) at University of Azad Jammu and Kashmir, Muzaffarabad.

Ethical statement

The study was conducted after approval from the ethics committee of the University of AJK, Muzaffarabad.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230726201056

Statement of conflict of interest

The authors of this research declare that there were no commercial or financial relationships that could be perceived as a potential conflict of interest during the conduct of the study.

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Supplementary Material

Hepatitis C Serological Markers Among the Apparently Healthy and Educated Population: Prevalence, Molecular Characterization, and Risk Factors Assessment

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Supplementary Table I. Students versus employee's based HCV prevalence among UAJ and K population.

Participants status	Number of individuals	ICT positive individuals	HCV RNA positive individuals
Students	5954 (85 %)	41 (0.69%)	16 (0.27%)
Employee	1061 (15%)	24 (2.24%)	10 (0.93%)
Total	7015 (100%)	65 (0.92%)	26 (0.37%)

Supplementary Table II. This table shows the detected viral load of HCV among participnts.

Viral load	No. of individuals	Percentage
Low viral load (few IU/ml to 50000IU/m)	9	35%
Intermediate viral load (50000-100000IU/ml)	5	19%
High viral load(higher than100000IU/ml)	12	46%

Supplementary Table III. This table shows the results of the LFTs detected for the PCR positive participants.

Tests	Normal range	No. of individuals within normal range	No. of individuals above normal range
Bilirubin	0.2-1.0 mg/dl	19 (73%)	7 (27%)
ALT	Male 0-43 U/L Female 0-36 U/L		
Alkaline Phosphate	Adult 80-306 U/L Child Up to 645 U/L		

Supplementary Table IV. This table shows the genotype distribution of HCV regarding gender.

Gender	Genotype 1a	Genotype 2b	Genotype 3a	Genotype 3b	Total
Males	4(23.53%)	3(17.65%)	8(47.05%)	2(11.76%)	17
Females	2(22.22%)	Nil	5(55.55%)	2(22.22%)	9

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0030-9923/2026/0001-0001 $ 9.00/0