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RESEARCH ARTICLE

Epidemiological and Genotypical Features of HCV in District Upper Dir, Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

Objectives: Objective: The incidence of Hepatitis C Virus (HCV) infection is high in Pakistan. The current study evaluates HCV risk factors, epidemiology, and genotype distribution in the less-developed Upper Dir Region, Khyber Pakhtunkhwa Province (KPK), Pakistan.

Methods: Blood samples from 500 outpatients from the Upper Dir Region were collected for initial screening using a strip-based immunochromatography test (ICT). In this study, ICT analysis followed Polymerase chain reaction (PCR) analysis for HCV-RNA confirmation. In addition, PCR positive samples were subject to genotypic description using genotype-specific primers.

Results: ICT analysis revealed that out of 500 patients, 13 (2.6%) were HCV positive. PCR analysis identified that 10 (2.0%) of the 13 HCV-positive patients were HCV-RNA positive. The overall incidence of HCV in the study group was 2.6%. The rate of HCV incidence was found to be higher in males (10/339 participants, 3.0%), while a lower incidence was found in females (3 of 161 participants, 1.9%). The patients between 45 and 60 years had the highest incidence rate (6 of 53 participants, 11.3%, $P<0.05$); while the patients between 25 and 44 years had an incidence rate of 4.2% (7 of 166 participants). Different HCV subtypes were also detected; 1a (two participants), 2b (one participant), 3a (two participants) and 3b (two participants). Three of the patients had untypable (UT) HCV genotype variants.

Conclusion: The study results suggest that; most of the cases were males and between 25 and 60-year-old. At the same time, several HCV subtypes are present in the inhabitants of the study area. Therefore, the presence of untypable genotypes may lead to diagnostic and therapeutic difficulties or failures. *J Microbiol Infect Dis 2021; 11(4):218-224.*

Keywords: *Genotypes, Hepatitis C Virus, incidence, Untypable*

INTRODUCTION

Hepatitis C is an infectious disease that causes liver inflammation. The Hepatitis C Virus (HCV) belongs to the Flaviviridae family of RNA viruses discovered in 1989 by Choo and his coworkers as the major cause of non-A and non-B hepatitis (NANB) [1]. It is an enveloped virus that has a genome size of 9.6kb flanked by an untranslated region (UTR) at both 5' and 3' ends. The HCV genome encodes 3008-3037 amino acids that are post-translationally processed to produce three

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structural proteins (SP) and six non-structural (NS) proteins [2].

The Prevalence of HCV varies from country to country [3]. The World Health Organization (WHO) estimates that global prevalence is approximately 3% (200 million people worldwide), with 3-4 million new infections annually [3,4]. Developing nations in Africa and Asia have the highest prevalence rates [5]. The pattern of HCV distribution is uneven in the Asia (4%) and Pacific regions (12%) [6]. Prevalence rates are highest in China (3.2%), Indonesia (2.1%), Saudi Arabia (1.8%) and India (1%) [7,8]. HCV infection rates in Europe are approximately 1% [9]. HCV infections spread rapidly in Pakistan, with approximately 10 million people (5% of the entire population) being infected [10]. In Pakistan, the distribution pattern of HCV is different in different geographical regions and even communities within a given area [11]. Many factors such as improper use of medical equipment by healthcare practitioners, needle sharing amongst drug abusers, non-hygienic practices in the service sectors (salons, barbershops, etc.), lack of public awareness, and unscreened blood transfusions contribute to the high prevalence rate of the infection in Pakistan [12].

Phylogenetically, six major genotypes of HCV have been reported based on nucleotide heterogeneity (35%). Each genotype is further divided into sub-genotypes, giving more than 100 sub-genotypes [13]. The dominant genotypes circulating across the globe are type 1 and 3. The most prevalent genotype in Pakistan is 3, with sub-genotype 3a being dominant [14]. Many studies have reported on the Prevalence of HCV in major Pakistani cities. However, none of these studies have reported HCV epidemiology, genotype distribution, and other covariates, such as age and gender in underdeveloped country areas. The current study aims to determine HCV incidence and genotype distribution in areas of Upper Dir. In addition, there is a general lack of HCV awareness due to poor education, low-socioeconomic conditions, and a lack of medical facilities.

METHODS

Ethical Consideration

All the experiments and procedures performed in this study were approved by the Ethics Committee of the Department of Zoology, Hazara University, Mansehra, Pakistan (Permit number: HU/2015/03/20/100A).

Study Area

This study was conducted in Upper Dir, Khyber Pakhtunkhwa, Pakistan (35° N latitude, 72° E longitude). Upper Dir spans an area of 3,699 km2 and has a population of approximately 0.946 million people, according to the 2017 census. This region was selected for the study owing to its unique socio-economic environment. However, the majority of the inhabitants have a lack education and awareness.

Blood Sampling

Blood samples from 500 patients who inhabitants of the Upper Dir Region were collected when they applied to the different local hospitals (Category D Hospital Wari, Rural Health Centre, Dislawar, Category D Hospital Chapar, DHQ Hospital, Upper Dir). Patients attending these hospitals with HCV-related symptoms, such as fatigue, poor appetite, itchy skin, bruising, yellow discoloration of the skin and eyes, etc., were included in this study. There were no age and gender restrictions in participation in the study.

Individuals who failed to provide the necessary details, such as information regarding their symptoms, associated risk factors, and contact information, were excluded from the current study.

Patients' personal data such as their name, age, gender, address, and contact information were recorded. The written consent was obtained from participants and/or the parents or guardians of participants. Blood samples were collected in proper tubes and labeled. Samples were spun down at 4000 × g for 10 minutes to obtain sera. The sera were appropriately stored under refrigeration at -20°C for further use.

Immunochromatographic Test (ICT)

For initial screening, a one-step Anti-HCV strip-based immunochromatographic test (ICT) (ACON® Lab. Inc., San Diego, CA 92121, USA) was used following the manufacturer's protocols to detect Anti-HCV antibodies [15]. In addition, ICT positive samples were subject to further molecular detection and genotype analysis.

HCV confirmation by PCR

Nested reverse transcription PCR (RT-PCR) was carried out using ICT positive samples for HCV-RNA detection as previously described [16]. Briefly, RNA was isolated from 250 µL of serum using Trizol reagent (15596018, Life Technologies) according to the manufacturer's instructions. The extracted RNA was stored at -80°C for RT-PCR. Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme (Invitrogen, Corp., California USA) was used for cDNA synthesis. A reverse primer (5'GTG CAC GGT CTA CGA GAC CT3') specific to the HCV genome was used for cDNA synthesis. The reaction mixture for cDNA synthesis containing nuclease-free water (3µL), 5X reaction buffer (4 µL), dNTPs (1µL), Moloney–Murine Leukemia Virus Reverse transcriptase (1µL), RNA template (10µL), reverse primer (1µL). The reaction mixture was kept in a thermal cycler for 1 hour at 42°C followed by 90°C for 2 minutes.

A 20 µl reaction mixture containing Taq DNA Polymerase (Fermentas Technologies USA) was prepared for Nested PCR for PCR confirmation of ICT positive samples. The amplified PCR product was run on 2% agarose gel for the detection of a specific HCV band. The positive samples were further subjected to qualitative PCR using an RNA quantification kit (Sacace, Biotechnologies, Italy) and Smart Cycler-II Real-time PCR machine (Cepheid, Calif, and Sunnyvale, USA).

HCV genotyping

HCV genotyping was carried out as described previously [17] using type-specific HCV primers to genotype all HCV-positive samples. Briefly, cDNA was synthesized using 100 U/µL of M-MLV (42°C, 60 min). Two microliters of cDNA were used in the first round of PCR to amplify a 470 bp HCV region (5'NCR along with core region). Two primers (SC2 and AC2) were used in this process. The reaction was carried out in 20 µl volume containing nuclease-free water (9.3 µl), 10x buffer (2 µl), MgCl2 (1.5 µl, 25 Mm), dNTPs (1µl, 2.5 Mm), Primer AS2 (1 µl), Primer AC2 (1 µl), DNA template (4 µl) and Taq polymerase (0.2 µl, 5 U/µL). The first-round PCR profile was; 94°C for 2 min (Pre-denaturation), followed by 30 cycles at 94ºC for 30 sec (denaturation), 45ºC for 1 min (annealing), and 72ºC for 1 min (extension). The final extension was 72 ºC for 10 min. The samples were stored at 4ºC until further use. The reaction was carried out using an automatic thermal cycler (ABIPCR system 2700). In the second round of PCR, two primers were used to identify the HCV genotypes. Two nested PCR reaction mixtures were prepared, each having two sets of primers (Table 1). Genotypes 1b, 2a, 2b, and 3b were identified using reaction mixture one, while genotypes 1a, 3a, 4, 5a, and 6a were identified using reaction mixture two. The second-round PCR profile was the same as the first. The amplified PCR product from the second round of PCR was run on a 2% agarose gel to identify a genotype-specific fragment.

Statistical analysis

SPSS statistical software version 16.0 was used for data analysis and tabulation. Statistical significance of the data was evaluated by Chi-square test, and the data were shown as mean values or number of patients. A p-value >0.05 was considered to be statistically significant.

RESULTS

Patient Demography

In total, 500 individuals were screened from the Upper Dir area. Of the 500 patients, 339 (67.2%) were male, and 161 (32.2%) were female. The participants were divided into different age groups; age group I (10-24 years) consisting of 267 (53.4%) individuals, age group II (25-44 years) consisting of 166 (33.2%) individuals, age group III (45-60 years) consisting of 53 (10.6%) individuals and age group IV (61-75 years) consisting of 14 (2.8%) individuals. Data analysis showed that the overall incidence of HCV recorded in Upper Dir during the current study was 2.6% (13 of 500 participants).

ICT and PCR Confirmation for HCV

All the blood samples were initially screened for anti-HCV antibodies by ICT. The results showed that out of 500 individuals, 13 (2.6%) tested positive. Although the antibody-based ICT is a good indicator of HCV, the 13 HCV-positive samples were further examined by PCR to detect HCV-RNA. Out of 13 samples, ten were found to be HCV RNA reactive, while three samples were found to be non-reactive. The viral load detected in the positive samples was in the range of 1.5 × 103 to 2.8 × 106 IU/ml.

Incidence of HCV by Gender

The incidence of HCV was found more prevalent among males (ICT, 3.0%) compared to females (ICT, 1.9%) (Table 2). PCR confirmation showed that 2.4% of males were infected, while only 1.2% of females were HCV positive. However, these differences were not statistically significant (P>0.05).

Incidence of HCV by Age

The next covariate that we analyzed was age (Table 3). Our analysis showed that age group III had the highest Prevalence (ICT, 11.3%; 7.5%, PCR), followed by age group II (ICT, 4.2%; PCR, 3.6%). HCV-positive individuals were not detected in the remaining two age groups (I&IV). Individuals in age group IV were asymptomatic. Statistical analysis revealed that the differences between the four age groups were statistically significant (P<0.05).

HCV genotypes circulating in the Upper Dir Region

PCR positive samples were genotyped to determine which were the most prevalent genotype circulating in Upper Dir (Figure 1). Out of samples, seven samples had type-specific bands, while three samples had UT genotypes. The incidence of the genotypes was as follows: 1a (two patients), 2b (one patient), 3a (two patients), 3b (two patients). Gender-wise genotypic distribution showed that out of 2 ICT positive females, 1 was UT, while the other had genotype 3a. In eight infected male subjects, 25% were UT, while the remaining had genotype 1a (25%), 2b (15.5%), 3a (12.5%) and 3b (25%).

DISCUSSION

Upper Dir is a relatively new district of KPK Province in Pakistan. This district is predominately mountainous and surrounded by forests. Poor infrastructure has left this area severely underdeveloped. In this study, we have determined the incidence and genotypic distribution of HCV among the natives of Upper Dir. This district was selected because of its prime geographical locations and lessening education resources to spread awareness among its inhabitants regarding HCV. Random sampling was carried out in a mixed population of individuals. Age restrictions were not applied to encourage participation.

The molecular epidemiology of HCV is different across different regions and groups [18]. In the general population of Pakistan, the seroprevalence of HCV is very high, approximately 6% [11]. The epidemiology of HCV recorded in the present study (2.6%) was comparatively less than that recorded in previous studies that focused on major cities, such as Islamabad (5.31%) [18], Faisalabad (20.89%) [19], Multan (4.06%) [18], Karachi (4-6%) [20], Mardan (9%) [21] and Bunir (5 %) [22].

A significant difference was observed in HCV incidence among different age groups. Age groups II & III were found to have a higher HCV prevalence compared to age groups I & IV. Interestingly, HCV-positive individuals in these groups went through dental surgery, identifying it as a potential risk factor. Unsterilized instruments for dental procedures in these poor areas is a likely source of infection. The high infection rates in adults can also be attributed to their frequent exposure to potential risk factors in their daily lives; for example, many patients regularly visited salons and barbershops where new blades were seldom used. The results of this study are consistent with previous reports, which suggest that HCV is highly prevalent among middle-aged individuals [23].

The HCV genome has high genetic variability, with a nucleotide substitution rate of 1.44×10^{-3} - 1.92×10^{-3} per site/year [24]. The high substitution rate in HCV results in different genotypes. Currently, six major genotypes and more than 100 subtypes have been identified. These diverse genotypes affect drug response in HCV-infected individuals [25]. Genotypes 2a and 3a are prevalent in the major cosmopolitan cities of various countries around the world [19]. Studies have shown that genotype 3a is the most predominant genotype in Pakistan (approximately 70%) [26]. Our genotypic analysis indicates that genotypes 1a (20%), 3a (20%), and 3b (20%) are equally prevalent among the people of Upper Dir. These results substantiate the findings of a previous study which shows that genotype 3a is the predominant genotype in Pakistan, followed by 1a and 3b [23, 27, 28]. In our study genotype, 2b was also detected in 1 infected person. This genotype is very rare in Pakistan, and its occurrence in the local population has been linked to the movement of people to North America, Europe, and Japan,

where it is frequently observed [29]. Interesting several UT genotypes were present in our study. UT genotypes have been previously reported in earlier studies carried out in Pakistan [30,23], indicating that it may be a growing healthcare issue in Pakistan. The higher occurrence of UT genotypes indicates that patients may have undergone interferon therapy or that the current genotype detection method failed to identify the genotype [25]. Conceivably, some novel genotypes may exist in Pakistan [23]. Novel, sensitive and reliable methods must be developed to detect untypable HCV genotypes.

Table 1. Oligonucleotide used for the 1st and 2nd round of PCR for HCV genotyping.

Primers	Sequence	Nucleotide Position
SC2	5-GGG AGG TCT CGT AGA CCG TGC ACC ATG-3’	24-3
AC2	-GAG(AC)GG (GT)AT(AG)TACCCCATGAG(AG)TCGGC–3’	417-391
Mix1		
S7	5-AGACCGTGCACCATGAGCAC-3’	12--18
S2a	5’-AACACTAACCGTCGCCCACAA-3’	40-60
G1b	5’-CCTGCCCTCGGGTTGGCTA (AG)-3’	222-203
G2a	5-CACGTGGCTGGGATCGCTCC-3’	178-159
G2b	5’-GCCCCAATTAGGACGAGAC-3’	325-306
Mix 2		
S7	5’-AGACCGTGCACCATGAGCAC-3’	12--18
G1a	5’-GGATAGGCTGACGTCTACCT-3’	196-177
G3a	5’-GCCCAAGGACCGGCCTTCGCT-3’	220-211
G4	5’-CCCGGGAACTTAACGTCCAT-3’	87-58
G5a	5’-GAACCTCGGGGGGAGAGCAA-3’	308-289
G6a	5’-GGTCATTGGGGCCCCAATGT-3’	334-315

Table 2. Prevalence of HCV by gender.

Gender	No. of Samples	Anti-HCV Antibody Positive Cases, n (%)	HCV-PCR Positive Cases, n (%)
Male	339	10 (3.0)	8 (2.4)
Female	161	3 (1.9)	2 (1.2)
Total	500	13 (2.6)	10 (2.0)

Table 3. Prevalence of HCV by age groups.

Age groups	Samples	Anti HCV Antibody Positive cases (%)	HCV PCR Positive cases (%)
10-24 years	267	0 (0.0)	0 (0.0)
25-44 years	166	7 (4.2)	6 (3.6)
45-60 years	53	6 (11.3)	4 (7.5)
61-75 years	14	0 (0.0)	0 (0.0)

This study has some limitations also. As many chronic HCV patients do not have specific symptoms until the disease has significantly progressed, this study cannot screen for such

individuals. Therefore, individuals suspected to be HCV positive were included in the study. Furthermore, HCV prevalence could only be examined in 500 participants due to a lack of funding. This also limited the number of variables that could be examined. These issues must be addressed better to understand HCV spread and infection in Upper Dir.

Conclusion

Hepatitis C infection is highly prevalent in Upper Dir. Residents of the area are unaware of the causes, symptoms, and preventive methods. The lack of education and knowledge contributes to the spread of the virus and may lead to higher mortality and morbidity rates in the future. Therefore, local authorities must take appropriate action to spread HCV awareness among the inhabitants in the district. This study has laid the foundation for future studies that must include more areas, participants, and molecular approaches to address the issue broadly.

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