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EPIDEMIOLOGICAL INVESTIGATIONS AND GENOTYPIC DISTRIBUTION OF HEPATITIS B AND C IN DISTRICT GILGIT, PAKISTAN

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Abstract

Hepatitis B and C is one of the major health care issues globally, mainly affecting developing countries like Pakistan. Over 2 billion populations were exposed to the Hepatitis B virus and 240 million are its chronic carriers with a 1 million annual mortality rate around the globe. However, the data related to Hepatitis B and C in district Gilgit, Pakistan is limited. Therefore, to investigate the seroprevalence and molecular characterization of Hepatitis B and C in district Gilgit, Pakistan. A total of 587 serums samples of patients were screened for HBsAg and anti-HCV antibodies by using the ICT strip method and those found positive were subjected to a polymerase chain reaction (PCR) for confirmation. Genotyping was based on PCR amplification using primers specific for HCV genotypes. The overall prevalence of HBV and HCV is 56% (329/587), among this HBV prevalence was higher at 84% (277/329) as compared to HCV at 16% (52/329). The predominant genotypes of HCV were 3a and 2a in Gilgit. Such a high prevalence of HBV and HCV in Gilgit, Pakistan is threatening. Therefore, it is necessary to monitor all the active cases of HBV and HCV in the district, of Gilgit, and conduct an urgent and efficient surveillance study in the remaining district of Gilgit Baltistan to limit the transmission of HBV and HCV.

Keywords

Hepatitis B, Hepatitis C, Nested PCR, Genotyping, Gilgit.

1. Introduction

Hepatitis C Virus (HCV) was first time isolated in 1989 as a causative agent of acute and chronic viral hepatitis. HCV is a positive sense enveloped RNA virus, that belongs to the Flaviviridae family (Ali *et al* ., 2010; Siddique *et al* ., 2020). However, the Hepatitis B virus (HBV) is a member of the hepadnaviridae family of the genus Orthohepadna virus (Schaefer, 2007). The mode of transmission of HCV and HBV is through sharing syringes by intravenous drug users, use of contaminated razors and toothbrushes, and sexual contact (Aziz *et al* ., 2010; Omran *et al* ., 2009). Hepatitis B and C is one of the major health care issues that the population around the world is facing, mainly affecting developing countries like Pakistan. An estimated over 2 billion populations were exposed to Hepatitis B virus among these 240 million people are its chronic carriers, which leads to 60% to 80% of liver cancer with a 1 million annually mortality rate worldwide (Ali SA, 2009; Koziel MJ, 2007; Oksuz Z, 2015; Waheed Y, 2009; WHO, 2017). Approximately, 8-20% of chronic infection of HBV is unevenly spread across the Arabian Peninsula, Asia, Egypt, the Pacific region, Alaska, and Sub-Saharan Africa. While, 2-7% of HBV chronic infection is reported from Eastern Europe, the Mediterranean basin, and North America. (Goldstein *et al* ., 2005; Zanetti *et al* ., 2008). As compared to HCV, HBV infection is 10 times more contagious disease, and as compared to HIV it was 50-100 times more contagious disease (Samuel D *et al*.,2004). The overall prevalence of HCV is 5% in Pakistan with approximately 10-17 million people being its chronic carriers (Aziz *et al* ., 2010; Siddique *et al* ., 2020). However, the frequency distribution of HCV is different in Punjab, Sind, Baluchistan, and KPK (Aziz *et al* ., 2010). HCV has six genotypes with more than a hundred subtypes. The genotypic studies of HCV were conducted for clinical management, vaccine development, and treatment of the HCV infection. The distribution of HCV genotypes is different among different geographical regions around the world. The predominant genotypes of HCV found around the globe were 1,2 and 3. Moreover, different subtypes were found in different regions. The dominant subtypes of HCV found in the USA and Europe are 1a and 1b (Idrees *et al* ., 2008; Nazir *et al* ., 2017; Nouroz *et al* ., 2015). While in Japan and China 1b is the most prevalent subtype of HCV (Yue *et al* ., 2010). In Pakistan 3a subtype of HCV was frequently found (Idrees & Riazuddin, 2008). However, the data related to the prevalence of HBV and HCV and the genotypic distribution of HCV in district Gilgit is not available. thus this study aimed to determine the prevalence of HBV and HCV and the genotypic distribution of HCV in district Gilgit.

2. Materials and Methods

2.1. Study Area

The current study was conducted in the district, of Gilgit, and a questionnaire was designed and presented to each individual before sampling to know information such as. Age, Sex, Address, and Received or Donated blood). The samples

were collected from different communities of Gilgit and were shifted to Provincial Head Quarter Hospital Gilgit (PHQ) for screening.

2.2. Samples Collection and Screening

A total of 587 samples (419 from Males and 168 from females) blood samples were collected. and screening for diagnosis of HBV and HCV in PHQ from respective areas. A 3 ml of blood sample was collected in EDTA and gel tubes and the serum was separated by centrifuging gel tubes at 5000 RPM. HBV and HCV initial screening was carried out by using rapid immune chromatographic (ICT) kits for the detection of HBsAg and anti-HCV.(Aziz *et al* ., 2010; Mujeeb & Pearce, 2008). Extraction of RNA/DNA of HBV and HCV: Qiagen DNA/RNA extraction kits were used for DNA/RNA extraction of ICT-positive samples of HBV and HCV. cDNA synthesis: From extracted RNA, complementary DNA (cDNA) is synthesized by using an RT enzyme by using a specific primer. (AS1: 5'-GTGCACGGTCTACGAGACCT-3') (Shafique *et al* ., 2009). cDNA amplification by PCR: For amplification of cDNA PCR was performed by using a specific primer as mentioned in table: 2. The thermos cycler conditions of PCR are, denaturation at 94ºC for 3 minutes (Denaturation), annealing at 55°C for 30 seconds (Annealing), and 72ºC for 30 seconds (Elongation) with 30 cycles. Nested PCR:For confirmation of HBV and HCV positive samples, PCR (Polymerase Chain Reaction) was performed by using specific primers mentioned in tables: 1 and 2. (Dettori *et al* ., 2009; Liew *et al* ., 2004). The amplified product was visualized on agarose gel electrophoresis. The 100 bp ladder (GeneRuler™, Thermo Fisher Scientific Inc., USA) was run along with amplifying product to determine the size of the amplicon.

Table 1: Primers are used in nested PCR for the detection of HBV

Genes	Primers	Annealing Temperature °C
B1-F:	CATCCTGCTGCTATGCCTCATCT	52 °C
B2-R:	CGAACCACTGAACAAATGGCA	
B3-F:	GGTATGTTGCCCCGTTTGTCCTCT	52 °C
B4-R:	GGCACTAGTAAACTGAGCCA	

Table 2: Primers are used in nested PCR for the detection of HCV.

Genes	Primers	Annealing Temperature °C
F-1:	5'-GCCATGGCGTTAGTATGAGT-3)	55 °C
R-1:	5'-GTGCACGGTCTACGAGACCT-3'	
F-2:	5'-GTGCAGCCTCCAGGACCC-3'	55 °C
R-2:	5'-CCGTGAGCGTTCGTGGGATA-3'	

2.3. Genotypic Identification of PCR Confirmed HCV Individuals

Multiplex PCR was performed for genotypic detection of HCV by using specific primers as described by (Ohno *et al* . (1997)) mentioned in table:03. The amplified product was visualized on agarose gels electrophoresis, and a 100 bp ladder (GeneRuler™, Thermo Fisher Scientific Inc., USA) was run along with amplifying product to determine the size of the amplicon. (Ahmadi Pour *et al* ., 2006; Ohno *et al* ., 1997)

Table 3: Specific primers are used for HCV genotypic detection

Target	Sequence	Nucleotide position	Amplicon Size
Sc2	GGGAGGTCTCGTAGACCGTGCACCATG	24–3	441
Ac2 1	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	417–391	-
Mix 1 S7	AGACCGTGCACCATGAGCAC	12–8	-
S2a	AACACTAACCGTCGCCCACAA	40–60	-
G1b	CCTGCCCTCGGGTTGGCTA(AG)	222–203	234
G2a	CACGTGGCTGGGATCGCTCC	178–159	39&190
G3b	CGCTCGGAAGTCTTACGTAC	164–145	176
Mix 2 S7	AGACCGTGCACCATGAGCAC	12–8	-
G1a	GGATAGGCTGACGTCTACCT	196–177	208
G3a	GCCCAGGACCGGCCTTCGCT	220–211	232
G4	CCCGGGAACTTAACGTCCAT	87–58	99
G5a	GAACCTCGGGGGGAGAGCAA	308–289	320
G6a	GGTCATTGGGGCCCCAATGT	334–315	346

Abbreviations used in the names of primers: S, sense; A or G, antisense; c, a core region

The notations 1a to 6a are following the HCV genotype nomenclature proposed by Simmonds.

3. Results

The results of the present study showed that, out of 587, 56 (9.54%) samples from both genders (Male and Female) were positive for HBV and HCV. Out of 419 samples from males, 33 (58.9%) were positive for HBV and HCV, and among 168 samples from females, 23 (41.07%) were positive for HBV and HCV as shown in Figure 1.

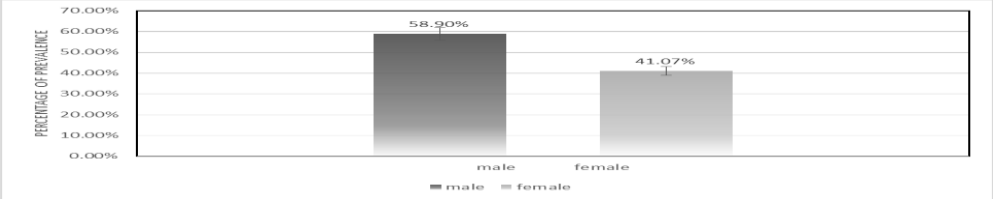

Figure 1: The overall prevalence of HCV and HBV in Males and Females in the district, Gilgit.

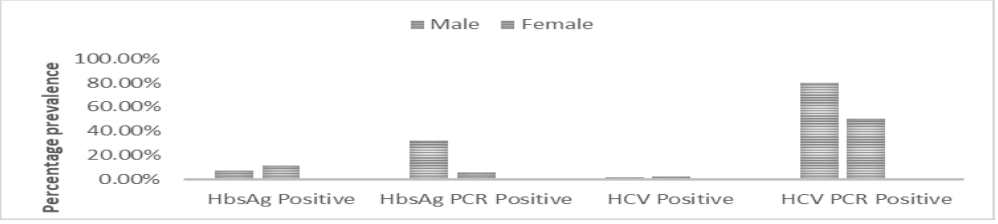

Figure 2: Prevalence of HBV and HCV in healthy individuals (Males and Females) was confirmed by PCR in the district, of Gilgit.

3.1 HBV Prevalence

The overall seroprevalence was 8% (47/587). of HBV was observed in both genders (Male and Female) A higher seroprevalence of HBV 11.30% (19/168) was found in females as compared to males 6.68% (28/419). Molecular conformation of 47 HBsAg positive samples of both genders (Male and Female) revealed that 21.27% (10/47) were positive in PCR. Out of 28 positive samples from males, only 32.14% (09/28) were positive in PCR, and out of 19 positive samples from females, 5.26% (01/19) were positive for HBV in PCR. However, only 10.52% (2/19) patients are symptomatic and 89.47% (17/19) are asymptomatic. However, a higher prevalence of HBV 44.7% (21/47) was found in the age group 21-30 years followed by the age group 10-20 years 40.42% (19/47) and 12.7% (6/47) was observed in the age group 31-40 years. The least prevalence of 2.12% (01/47) is observed in the age group 41-50 years, as mentioned in table 4.

Table 4: Prevalence of HBV in different age groups in the population of the district, Gilgit.

Age group(years)	HBV-antibody positives		HBV-PCR positives	
	Positives	Percentage %	Positives	Percentage %
10-20	19	40.42%-	10	52.63%
21-30	21	44.7%	07	33.33%
31-40	06	12.7%	02	33.33%
41-50	01	2.12%	-	50%
51-60	-	-	-	-

3.2 HCV Prevalence

The overall seroprevalence was 1.53% (09/587). of HCV was observed in both genders (Male and Female) A higher seroprevalence of HCV 2.38% (04/168) was observed in females as compared to males 1.19% (05/419). Molecular conformation of 09 anti-HCV positive samples of both genders (Male and Female) revealed that 66.66% (06/09) were positive in PCR. Out of 05 positive samples from males, 80% (04/05) were positive in PCR, and out of 04 positive samples from females, 50% (02/04) were positive for HCV in PCR. However, only 33.33% (02/06) patients are

symptomatic and 66.66% (04/06) are asymptomatic. However, the higher prevalence of HCV 44.4% (04/09) was observed in the age group 21-30 years followed by the age group 31-40 years 33.3% (03/09), and least prevalence of 22.2% (02/09) is observed in the age group 41-50 years, respectively. mentioned in table 5.

Table 5: Prevalence of HCV in different age groups in the population of the district, Gilgit.

Age group(years)	HCV-antibody positives		HCV-PCR positives	
	Positives	Percentage %	Positives	Percentage %
10-20	-	-	-	-
21-30	04	44.4%	03	75%
31-40	03	33.3%	02	66.66%
41-50	02	22.2%	01	50%
51-60	-	-	-	-

3.3 Genotypic Study of HCV

A total of 06 HCV PCR confirm samples were further employed for the Genotypic study. The multiplex PCR results revealed that the predominant genotypes of HCV in Gilgit are 2a and 3a. The prevalent genotype found in males was 2a and, in females was 3a.

Table 6: The pattern of HCV genotype among the infected patients (N = 06).

Genotype	Subtype	Male (%)	Female (%)	Total (%)
Genotype 2	2a	04 (100%)	-	04 (66.66%)
Genotype 3	3a	-	02 (100%)	02 (33.33%)
Total	-	04	02	06

4. Discussion

Hepatitis B and C are major public health problems around the world. Mainly, targeting developing countries like Pakistan (Ali *et al* ., 2010; Shafique *et al* ., 2009). According to the World Health Organization, almost 3% population is affected by hepatitis C worldwide, with an increased rate of 3 to 4 million cases annually, Hepatitis C cases in the world are 170 million (Raza *et al.*, 2007). However, in a third-world country like Pakistan, an estimated prevalence of HBV carriers is about 7-9 million, with a carrier rate of 3-5%. It is very necessary to know the frequency distribution of a particular disease in a specific area. This is important to provide better clinical management of a disease. This study was the first one to be held in Gilgit-Baltistan to know the epidemiology and molecular characterization of hepatitis B and hepatitis C in the district, of Gilgit. The outcome

of the current study revealed that overall seroprevalence was 8% (47/587). (Manzar *et al* . (2003)) conduct a study on naval recruits of Pakistan armed forces, and their results showed that the seroprevalence of HbsAg was 3.2% and HCV was 2.2%. In another study conducted by (Manzoor (1997)) in northern areas of Pakistan, according to this study, the overall seroprevalence of HBV was 37%. In our study, a higher seroprevalence of HBV 11.30% (19/168) was found in females as compared to males 6.68% (28/419). Molecular conformation of 47 HBsAg positive samples of both genders (Male and Female) showed that 21.27% (10/47) were positive in PCR. Out of 28 positive samples from males, only 32.14% (09/28) were positive in PCR, and out of 19 positive samples from females, 5.26% (01/19) were positive for HBV in PCR. A study related to our study was conducted by (Rauf *et al* . (2011)), which showed that the seroprevalence of hepatitis B and C was 9.7%. Among these 4.6% was confirmed by PCR. However, 56% of individuals were asymptomatic, and the predominant genotypic found in males was 3a (Afridi *et al* ., 2009). Currently, a higher prevalence of HBV 44.7% (21/47) was found in the age group 21-30 years followed by the age group 10-20 years 40.42% (19/47) and 12.7% (6/47) was observed in the age group 31-40 years. The least prevalence of 2.12% (01/47) is observed in the age group 41-50 years. The outcome of a study conducted by (Rauf *et al* ., 2011), shows that, out of 29 individuals, 5 are positive for HBV antibodies, out of 5,2 individuals belong to the age group 36-45 years and 1 belongs to each age group in 16-25, 26-35 and 46-60 years. The findings of our study were also favored byAltaf Ahmed *et al* . (2006) and showed that the age-wise distribution of Hepatitis B and C infection was greater in young persons above the age of 20 in Pakistan. In the current study, overall seroprevalence was 1.53% (09/587). of HCV was observed in both genders (Male and Female) A study conducted by (Alam *et al* . (2021)), also reported a higher seroprevalence of 8% of HCV in healthy individuals of the district, Gilgit. Previously, few studies, showed that the prevalence of HCV was 8.64% and 6.2% general population of Pakistan (Arshad & Ashfaq, 2017). In our study, a higher seroprevalence of HCV 2.38% (04/168) was observed in females as compared to males 1.19% (05/419). Molecular conformation of 09 anti-HCV positive samples of both genders (Male and Female) revealed that 66.66% (06/09) were positive in PCR. Out of 05 positive samples from males, 80% (04/05) were positive in PCR, and out of 04 positive samples from females, 50% (02/04) were positive for HCV in PCR. However, the higher prevalence of HCV 44.4% (04/09) was observed in the age group 21-30 years followed by the age group 31-40 years 33.3% (03/09), and least prevalence of 22.2% (02/09) is observed in the age group 41-50 years. A study conducted by Siddique *et al* . (2020) revealed that 0ut of 172 (5.57%) HCV ICT positive samples 55.8% (96/172) were confirmed by PCR. However, according to this study, the 41-50 years' age group showed high HCV prevalence

followed by the age group 31-40 years 22.9%. A similar, result is also reported in a study conducted by (Ahmad *et al* ., 2010), and showed a higher prevalence of HCV in the age group 21-40 years. Moreover, a study conducted in the USA reported a higher prevalence of HCV in the age group 30-49 years (Alter, 2007; ArmstrongGL *et al* ., 2006). Due to a lack of a proofreading mechanism in HCV. HCV has 7 major genotypes with more than 100 sub-genotypes. (Smith *et al* ., 2014). In our study, a total of 06 HCV PCR confirmed samples were further employed for the Genotypic study. The multiplex PCR results revealed that the predominant genotypes of HCV in Gilgit are 2a and 3a. The prevalent genotype found in males was 2a and, in females was 3a. However, similar results are also reported by (Siddique *et al* . (2020)) and show that 3 is a prevalent genotype found in Faisalabad, Pakistan. Several studies from Islamabad, Faisalabad, and Rawalpindi also reported that HCV 3 genotype is prevalent in these cities of Pakistan (Afzal, 2017; Ahmad *et al* ., 2007). Moreover, multiple studies also reported a high percentage of HCV genotype/ subtype 3a (Ahmad *et al* ., 2007; Ahmad *et al* ., 2010; Butt *et al* ., 2010; Idrees *et al* ., 2009; Waheed *et al* ., 2009; Waqar *et al* ., 2014). Therefore, large-scale studies involving different districts of Gilgit Baltistan should be conducted on a mass scale to find out the seroprevalence of Hepatitis B and C and for understanding the molecular epidemiology of the predominate genotypes in the region.

5. Conclusions

Such a high prevalence of hepatitis B and C in the district, of Gilgit is alarming. The outcomes of this investigation illustrate the significant occurrence of HCV and HBV in males as compared to females in the study area. Genotype 3a among females and 2a among males is the redominant strain of HCV found in the district, of Gilgit. However, it is necessary to conduct studies on large scale to determine the frequency distribution of Hepatitis B and C genotypes in the remaining nine districts of Gilgit Baltistan to adopt better clinical management of the disease.

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