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Prevalence of Hepatitis B and C Virus in the General Population of Hill Surang Area, Azad Jammu and Kashmir, Pakistan

Abdul Rauf,[1] Muhammad Shahid Nadeem,[1] Muhammad Arshad,[2] Haroon Riaz,[1] Muhammad Muzammil Latif,[2] Muhammad Iqbal,[2] Muhammad Zahid Latif,[2] Nisar Ahmed[3] and Abdul Rauf Shakoori[1,*]

[1]*School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan*
[2]*Healthcare Diagnostics and Research Centre, Allama Iqbal Town, Lahore, Pakistan*
[3]*Human Appeal International, I-8/4 Islamabad Pakistan.*

Abstract.- Present study was conducted to investigate the prevalence of hepatitis B and C viruses (HBV, HCV) among the general population of a village population in the Azad Jammu and Kashmir area in Pakistan. Randomly selected male and female individuals of 5 to 70 years of age were screened for anti-body test for HCV and HBV. The samples found positive for virus related antigen and antibody were further investigated for the presence of viral RNA and DNA in the plasma using polymerase chain reaction (PCR). HCV positive samples were further analyzed for the genotypes. Total 520 individuals (250 males and 270 females) from general population who willingly participated in the screening process were included in the study. Prevalence and the type of virus, the age, sex, history of exposure and the previous surgical of the participants was also investigated. 7.5% of participants were found positive against HCV antibody and only 0.96% were positive for HBV surface antigen. Among the individuals positive for HCV-PCR, the genotype 3a was the most prevalent, whereas genotypes 2a, 1b, 3b and an unidentified strain were also found. It is concluded that PCR based analysis along with concomitant HBsAg and anti-HCV testing permits diagnosis HBV and HCV infections.

Keywords: Hepatitis B and C, HBV, HCV, HBsAg.

INTRODUCTION

Viral hepatitis and hepatocellular carcinoma are world-wide health problem. The possibility of hepatitis transmission through blood and other body fluids are known since long (Mahoney, 1999; Hillyer *et al.*, 2001). Hepatitis B virus (HBV) is one of the major health problems in the world (Khan and Riazuddin, 2004; Zhu *et al.*, 2008; Ali *et al.*, 2011), the condition is severe in Asia, southern Africa, Latin America and Europe. According to the estimates more than 2 billion people are infected with HBV world-wide (Paraskevis *et al.*, 2002; Li *et al.*, 2010). Among these patients about 400 million are suffering from chronic HBV (Alam *et al.*, 2007). Pakistan is highly endemic with HBV (Noorali *et al.*, 2008). Recent findings indicate that 9 million people are infected with the disease and there is a rapid increase in the rate of infection (Hakim *et al.*, 2008). HCV is the other viral infection of liver which is associated with greater mortality (Fabrizi *et al.*, 2004, 2007; Butt *et al.*, 2007) because the hepatocellular carcinoma and liver cirrhosis are more common among anti-HCV-positive patients (Henderson *et al.*, 2010). According to the recent estimates of the World Health Organization (WHO), HCV infection has infected about 170 million people (some 3% of the world's population) (George *et al.*, 2001; Higuchi *et al.*, 2002). There are several genotypes of HCV reported in the literature with nine well studied genotypes (Ohno *et al.*, 1997).

A large proportion of Pakistani population is unaware about the epidemiology and risk factors of viral hepatitis. Although the screening and diagnostic recommendations advocate early detection of HBV and HCV (Lok and McMahon, 2007) but most of the victims of viral infections are brought to the hospitals in Pakistan when they are at the end stages of liver damage. The late diagnosis increase the risk of hepatocellular carcinoma and decrease the effect of antiviral therapies (Strader *et al.*, 2004; Lok and McMahon, 2007; Di Bisceglie, 2009). The present study was, therefore, aimed to

* Corresponding author: arshaksbs@yahoo.com
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investigate the general population for the viral hepatitis infections.

MATERIALS AND METHODS

A free hepatitis screening camp was arranged by the combined sponsorship and intellectual manpower collaboration of Human Appeal International and researchers of the School of Biological Sciences, University of the Punjab Lahore Pakistan, in the Hill Surang area of Azad Jammu and Kashmir. The screening of viral hepatitis B and C for antigen and antibody has been carried out in the general male and female population, who voluntarily offered the blood sampling. More than 800 people were offered the hepatitis screening, but only 520 (250 males and 270 females) from general population of 5 to 75 years of age were screened for HCV antibodies and HBV antigen. For primary screening of anti HCV and anti HBsAg, screening kits (ICT:ACON®, ACON Laboratories Inc., San Diego, CA 92121, USA) were used.

Three drops of separated serum were taken by the dropper provided in the kit and placed on the kit device. The sample showing two bands against C (control sample) and T (test sample) were considered positive for the hepatitis B surface antigen. For HCV screening a drop of serum was placed in the sample space in the kit device and three drops of buffer, provided with the kit, were added to it. The samples showing two bands against both C and T were considered positive for anti-HCV antibody. The individuals appearing positive in the screening were further subjected to PCR based detection of viral DNA/RNA and its correlation with different diseases and risk factors were also studied.

For the confirmation of HBV DNA the serum samples from antigen positive male and female individuals were used for DNA isolation with DNAzol® BD using the procedure described by the manufacturer. Isolated DNA was used for PCR reaction using following primers in the first round

5’-catcctgctgctatgcctcatct-3’ and
5’-cgaaccactgaacaaatggcact-3’.

The PCR product of the first round was then used as template for the nested PCR based amplification using the following primers

5’-ggtatgttgcccgtttgtcctct-3’ and
5’-ggcactagtaaactgagcca-3’

The second round primers were selected after the inner regions of the DNA fragment amplified in the first PCR reaction. The reaction mixture for the first and second round contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 ρmol of both primers, and 2.5U of Taq polymerase. The thermocycler was adjusted at 94°C for denaturation for 2 min, followed by 30 cycles, each of 30 seconds of denaturation at 94°C, annealing for 40 sec at 53°C and 30 sec at 72°C with final extension at 72°C for 1 min. Both rounds of PCR were carried out under the same PCR conditions.

For the confirmation of hepatitis C virus in the blood plasma, total RNA was isolated from the plasma of individuals positive for HCV antibody with Trizol-reagent kit (Invitrogen), the RNA was isolated according to the procedure as described by the manufacturer. Single stranded complementary DNA (cDNA) was generated using sequence-specific reverse primers using reverse transcriptase polymerase (M-MuLV-Moloney murine leukemia virus) and used for PCR amplification. In the first round of PCR-amplification, the primers with nucleotide sequence of

5’-ccctgtgaggaactactgtcttcacgc-3’ and
5’-actcgcaagcaccctatcaggcagtac-3’,

whereas the primers used for amplification of the internal region include:

5’-gaaagcgtctagccatggcg-3’ and
5’-cacaaggcctttcgcgacc-3’.

The thermocycler was adjusted at 94°C for denaturation for five minutes, followed by 30 cycles, each of 30 sec of denaturation, 40 sec annealing at 64°C and 30 sec at 72°C of extension with final extension at 72°C for 1min. The nested PCR was carried out under the same conditions with an annealing temperature of 53°C. The PCR positive

Table I.- The details of primers for genotyping of HCV as described by Ohno *et al.* (1997).

Primer*	Sequence (5'–3') nucleotide	Expected position	Band size (bp)
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	139&190
G2b	GGCCCCAATTAGGACGAGAC	325–306	337
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, core region. The notations 1a to 6a are in accordance with the HCV genotype nomenclature proposed by Simmonds *et al.* (1994).

samples were investigated for the genotype of HCV, a modified procedure based on the information provided by Ohno *et al.* (1997). A single strand of viral complementary DNA (cDNA) was generated with reverse transcriptase (M-MuLV) using core-gene specific reverse primer using total RNA as template. The genotypes of HCV were identified with multiplex-PCR reactions containing two set of primers each processed in parallel in the nested PCR. The details of primers and the size of PCR amplified fragments are shown in Table I.

PCR-thermocycler was adjusted at 94°C for denaturation for 2 min, followed by 30 cycles of each of 30 sec denaturation at 94°C, 40 sec annealing at 53°C and 30 sec extension time at 72°C. The nested PCR was carried out under the same PCR conditions as above. The expected sizes of the genotype-specific bands amplified by PCR typing are as follows: genotype 1a, 208 bp; genotype 1b, 234 bp; genotype 2a, 139 bp and 190 bp (note that, theoretically, the 190-bp amplicon may also be detected in a small proportion of HCV type 4 isolates); genotype 2b, 337 bp; genotype 3a, 232 bp; genotype 3b, 176 bp; genotype 4, 99 bp; genotype 5a, 320 bp; and genotype 6a, 336 bp.

RESULTS

Anti-HCV and Anti-HBsAg positive cases

Out of 520 individuals, 39 (7.5%) were found positive against HCV antibody test and only 5 (0.96%) were positive for HBV surface antigen.

HCV genotypes

The samples found positive for antibody and antigen based screening, were further investigated for the presence of hepatitis C related RNA and hepatitis B related DNA with PCR. Hepatitis C viral RNA was detected from the plasma of 17 (43.6%) antibody positive individuals (Fig.1). Out of 17 HCV-PCR positive samples 6 (35.3%) were symptomatic and 11(64.7%) were asymptomatic, 12 (70.5%) of the PCR positive samples were females and 5 (29.5%) were male. Among the HCV positive samples, genotype 3a was the most dominant form in 6 (50%) of the PCR positive females and 2 (40%) of PCR positive male subjects (Table II). The other genotypes detected in the population included 3b in one female (8.3%) and one male (20%); 2a was found in two females (16.7%) and not detected in the male subjects; and1b was found in two females

Table II.- Hepatitis C Prevalence in different age groups of healthy population of Hill Surang Azad Kashmir Pakistan.

Age groups (years)	Anti-HCV positive	HCV PCR positive	Anti HCV (%)	HCV PCR (%)	Anti-HBsAg positive	HBV PCR positive	Anti HBsAg (%)	HBV PCR (%)
5-15	2	0	5.13	0	0	0	0	0
16-25	7	0	17.94	0	0	0	0	0
26-35	9	6	23.07	35.29	0	0	0	0
36-45	10	8	25.64	47.06	3	1	60	50
46-75	11	3	28.20	17.65	2	1	40	50

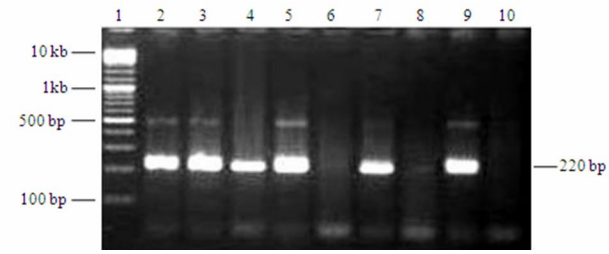

Fig. 1. Detection of HCV through PCR amplified cDNA run on 2% agarose gel. Lane **1,** 10kb DNA marker (Fermentas Cat#SM0334); Lanes **2, 3, 4, 5, 7** and **9:** positive for HCV; Lanes **6, 8, 10** are negative for HCV.

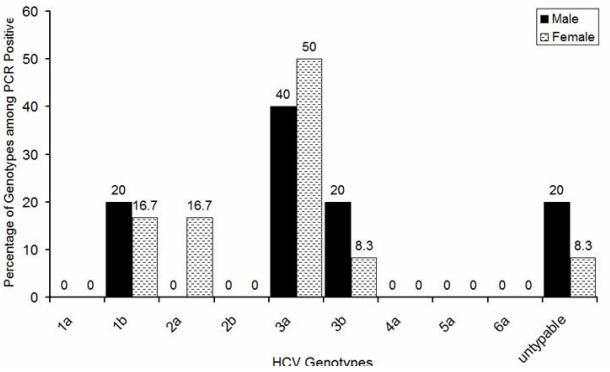

Fig. 2. Hepatitis C genotypes in male and female subjects. A. In female subjects 3a genotype was found to be 50%, 2a and 1b 16.7% each, 3b 8.3% and untypable 8.3%. The genotypes 1a, 2b, 4a, 5a and 6a were not detected. B. In male subjects, genotype 3a was 40%, 1b and 3b 20% each and untypable 20% whereas the strains 1a, 2a, 2b, 4a, 5a and 6a were not detected.

(16.7%) and one male (20%); one females (8.3%) and one male subjects (20%) were found positive with an unknown genotype (Fig.2). The genotypes 1a, 2b, 4a, 5a and 6a as described in the population studies by Ohno *et al.* (1997) were not detected in the PCR positive samples described in the present study.

HBV DNA analysis

Among 5 Anti-HBsAg positive samples 2(40%) were found positive for viral DNA. Both were asymptomatic males. DNA for hepatitis B is shown as a band at 119 bp on agarose gel electrophoresis (Fig. 3).

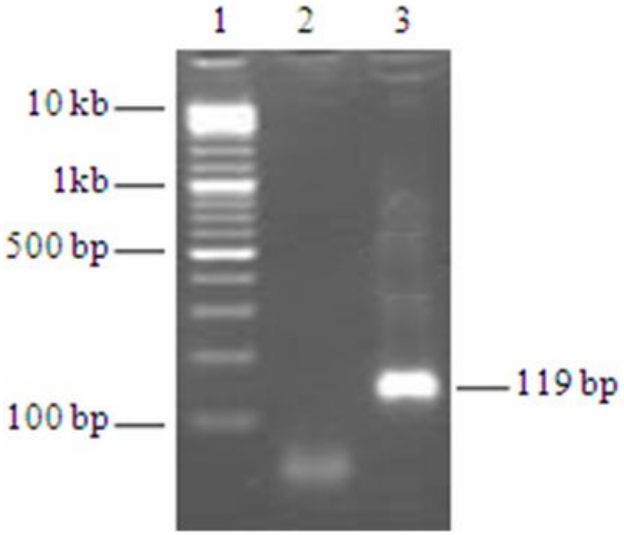

Fig. 3. A representative 2% agarose gel showing HBV positive band in lane 3. The lane 2 has HBV negative sample. The lane 1 shows Fermentas 100-10,000 bp marker

Frequency of hepatitis among different age groups

The individuals of 5 to 75 years of age were investigated in the present study. Among the age groups of 5-15, 16-25 and 26-35 years, respectively

2, 7 and 9 were found positive for anti-HCV; there was no anti-HBsAg positive (Table II). In the age group of 36-45 years, 10 were found anti-HCV positive and 3 were found positive for anti-HBsAg, out of these positive samples 8 and 1 were found positive for HCV and HBV related PCR based diagnosis respectively. In the last age group, 46-75 years, 11 were found positive for anti-HCV out of which 3 were found positive for HCV-PCR, two were anti-HBsAg positive and 1 of them was HBV-PCR positive.

DISCUSSION

The prevalence of HBV and HCV infections determined by random sampling of male and female subjects and screening for HBsAg and anti-HCV is described in our study of general population. The samples found positive in the preliminary screening were further investigated for the presence of DNA and RNA of HBV and HCV in the blood plasma. The prevalence of HCV infection was 7.5% in the anti-body screening and 0.96% HBV infected samples were detected. Hepatitis C 3a genotype was the most predominant in male and female individuals along with the well established genotypes and an unknown genotype. The results suggest a hypothesis about the presence of some HCV genotypes in Pakistan which are different from those described in studies conducted in Asia, Europe and USA and being used as the basis for the diagnostic methods in the clinical laboratories in the country (Ohno *et al.*, 1997). This hypothesis is also supported by the studies conducted in Pakistan by various groups in the past (Idrees and Riazuddin, 2008; Ahmad *et al*., 2010; Attaullah *et al*., 2011). The unknown genotype is detected in a considerable percentage in the Pakistani population and it is a matter of considerable importance and challenge for the local researchers to determine the complete genome sequence(s) of these genotypes and optimize the procedures for the early diagnosis of related infection. The incidence rate of viral hepatitis described in the present study is less than the studies of some populations from various areas of the world (Darwish *et al.*, 1995; Sarkari *et al.*, 2012). However, the rate of infection is considerable for the action of related authorities. Our results have shown that the hepatitis caused by HBV is higher in male individuals whereas the infection caused by HCV is higher in female population. The detailed histories of positive subjects have shown that the blood transfusion and surgical operation are the major risk factors. Among 17 HCV positive female subjects confirmed by PCR, 6 (35.2%) were found to be infected during cesarean and other surgical operations. Two male individuals were found positive for both HBV and HCV.

The study presented in this manuscript describes the status of viral hepatitis infection in the general population in an area with less environmental pollution and contamination in the food stuff which indicates an alarming condition of hepatitis specially HCV infections in the general population and need for the country-wide screening of population to diagnose the disease at early stages and prevent the threats to human life.

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