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Prevalence and Molecular Determination of Hepatitis C Infection in Khyber Pakhtunkhwa, Pakistan

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Background: Hepatitis C virus (HCV) infection is the most significant source of chronic liver diseases in the globe. About 170 million individuals are infected by HCV worldwide. The reported prevalence of HCV in different areas of Khyber Pakhtunkhwa (KP) ranges from 4.1 to 36%.

Objectives: The current study aimed to analyze the true prevalence of HCV infection in Khyber Pakhtunkhwa, Pakistan.

Materials and Methods: Three hundred and ninety HCV enzyme-linked immunosorbent assay (ELISA) positive samples that belonged to the different regions of Khyber Pakhtunkhwa were sent to the Genome Centre for Molecular Based Diagnostics & Research (GCMBDR), Lahore, from January 2011 to March 2011, and were selected for the current study. Serological and biochemical data of these samples were provided by clinicians. Out of 390 samples, 40 were provided from Mardan, 65 from Dir (Lower), 185 from Swat and 100 from Malakand districts of Pakistan.

Results: Out of 390 patients, 140 were found HCV RNA positive (by Polymerase Chain Reaction method) and 250 subjects were excluded from further analysis. Out of PCR positive subjects, 81 were male and 59 were female. All individuals were categorized in four age groups that is, 0 to 20, 21 to 40, 41 to 60 and above 60 years. HCV RNA was found in 16.67%, 37.5%, 35.51% and 36% of these groups, respectively. District wise HCV positivity rates were 36.2 % in Swat, 38.4% in Dir (L), 36% in Malakand and 30% in Mardan, respectively.

Conclusions: It was found that among the studied areas, Dir district had the highest prevalence of HCV, the majorly of affected patients were among the age group of 21 to 40 , male patients were found more susceptible to this infection ($P = 0.0103 < 0.05$), and the possible reason can be the high exposure of males to the HCV infection risk factors. Furthermore the current study was unable to find the important risk factors responsible for the frequent prevalence of HCV infection in Khyber Pakhtunkhwa

Keywords: Hepatitis C Virus; Prevalence; Gender; Disparity; Khyber Pakhtunkhwa

1. Background

Hepatitis C virus (HCV), a member of Flaviviridae family (genus Hepacivirus) is mostly responsible for chronic hepatitis and hepatocellular carcinoma (1, 2). Over 170 million individuals are infected with HCV around the world (3). The reported rate of HCV in Syria, Saudi Arabia and Jordan is 1%, 2.5% and 1.7%, respectively (4-6). HCV has a positive sense RNA and is about 50 nm in diameter (1, 7). Targeted site of HCV is hepatocytes of the liver that may start replication there. It is reported that about fifty virions are produced by each infected cell per day. Hepatitis C virus may also replicate in peripheral blood mononuclear cells, which may lead to high level of immunological disorders in chronically infected HCV patients. Due to RNA virus and lack of proof reading ability, mutation rate is very high in HCV genome. This high mutation rate is responsible for the existence of different genotypes (8). Six primary genotypes and several subtypes are known globally (9). Genotypes 1, 2 and 3 are circulated worldwide while the majority of subtypes 1a and 1b have been reported in Europe and the United States of America (9-13). In North America, Japan, and Europe the most frequently found subtypes are 2a and 2b while 2c is very common in Italy (10-13). HCV genotype 4 is mostly reported in Middle East and North Africa (14, 15), while genotypes 5 and 6 are only distributed in Hong Kong and South Africa (16,

17). Transmission of HCV from the infected to healthy individuals may occur by different ways such as contaminated syringes, sharing contaminated tooth brushes, razors etc. It may also be transmitted by sexual contact with infected persons (18). Hepatitis C virus was also found in saliva and breast milk, but a transmission through breast milk has not been reported (19). Transmission via needle stick injury is documented, about 5% (20). Different diagnostic methods are used to detect HCV including ELISA, recombinant immunoblot assay, and polymerase chain reaction (PCR). PCR is considered to be the most accurate and reliable method that can detect the virus after one to two weeks of infection while by antibodies it may take a long time (21-23). No vaccines are available for protection against HCV (24). About 50% to 80% of the infected patients get chronic infection and 40% to 80% of these patients may clear their body from infection via treatment (25-27). Patients with chronic HCV infection should be vaccinated against Hepatitis A virus (HAV) and Hepatitis B virus (HBV) infections and should avoid the use of alcohol and liver toxic agents (22). Different antiviral drugs are used to treat HCV (28). Depending on HCV genotypes, 24 or 28 week treatments including interferon alpha (pegylated in some cases) and ribavirin are currently in practice (22). Various other therapies are claimed to be helpful in HCV treatment such as milk thistle, and colloidal silver and ginseng, but no valid scientific evidence for the efficacy of these agents on the virus has been reported (29). Hepatitis C virus prevalence range in different geographical regions of Khyber Pukhtunkhwa (KPK) has been reported from 4.1 to 36% in (30, 31).

2. Objectives

The current study aimed to analyze the prevalence of HCV infection in Khyber Pakhtunkhwa, Pakistan using conventional PCR and real time PCR.

3. Materials and Methods

3.1. Sample Collection

Three hundred and ninety HCV ELISA positive samples were collected from January 2011 to March 2011 in different war affected areas of KPK that is Peshawar, Mardan (40), Malakand (100), Dir (L) (65) and Swat (185) districts. Samples were processed at GCMBDR Lahore. All sera were stored in aliquots at -70°C till used for nucleic acid (RNA) extraction. Out of 390 samples, 140 were HCV RNA positive (in 3 to 70 years old group) among which 81 were male and 59 were female serological and biochemical data of these patients were recorded; and 250 subjects were excluded from further data analysis

3.2. Isolation of Hepatitis C Virus RNA From Samples

Hepatitis C virus RNA was isolated from 100 μL serum sample by Ana-gen RNA extraction kit (Ana-gen, USA) according to the manufacturer's instructions.

3.3. cDNA (Complementary DNA) Synthesis

From the isolated RNA, cDNA was synthesized by 100 U of Malany-murine leukima virus (MMLV) reverse transcriptase enzymes. Reagents used in cDNA synthesis were 4.0 μL of 5× first strand buffer, 0.5 μL 0.1 MDTT, 2.0 μL dNTPs (10 mM), 1.0 μL anti-sense primer, and 1.5 μL dH 20. The final volume was adjusted up to 20 μL. Cycling profile for cDNA synthesis was 37°C for 50 minutes, 94 °C for two minutes and 20°C for two minutes. Two rounds of PCR were performed after cDNA synthesis.

3.4. First Round Polymerase Chain Reaction

For qualitative detection of HCV RNA, RT-PCR method was used according to Idrees protocol . In the first round of PCR, 5` UTR region was amplified by 2 μL of synthesized cDNA as a template. Reagents used in the first round were as follows: 2 μL 10× PCR buffer containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.2 μL of MgCL2, 1 μL of dNTPs, 1 μL of outer sense primers (GGC GAC ACT CCA CCA TGG A), 1 μL of outer anti-sense primers (TTG CAC GGT CTA CGA GAC C), 1 μL taq DNA polymerase and 8.8 μL of dH2O. Cycling profile for the first round of PCR was as follows: initial denaturation at 95°C for two minutes, followed by 30 cycles of 94°C for 45 seconds, 54°C for 45 seconds, and 72°C for one minute. Final elongation was done at 72°C for 10 minutes. PCR product was stored at 4°C for further processing.

3.5. Second Round Polymerase Chain Reaction

Reagents used in the second round of PCR were the same as in round one, except that the inner set of primers were used instead of the outer sense primers; with the following sequence: TCA CTC CCC TGT GAG GAA CT and TCC CGG GGC ACT CGC AAG CA at the annealing temperature of 50°C. PCR product of the first round was used as a template.

3.6. Gel Electrophoresis

The second round PCR products were electrophoresed on a 2% agarose gel prepared in 1 × Tris-borate-EDTA (TBE) buffer, and stained with ethidium bromide. Then the products were observed under the UV transilluminator. The sizes of PCR products were estimated according to the migration pattern of a 50-base pair (bp) DNA ladder (Fermentas Life Sciences Co.).

4. Results

4.1. Prevalence of Hepatitis C Virus Infection in Both Sexes and Various Age Groups

Totally, out of the 390 subjects participating in this study, 140 were found positive for HCV RNA by PCR, out of which 81 (40.5%) subjects were male, and 59 (31.05%) female. The rate of HCV infection was higher in male subjects compared to female ones (Table 1).

All PCR positive patients were divided into four age

groups that is 0 to 20, 21 to 40, 41 to 60 and above 60, and HCV infection was significantly higher in patients of 21 to 40 years old age group. Hepatitis C virus was detected in 16.67%, 37.5%, 35.51%, 36% patients of each group respectively (Table 2). The prevalence of ELISA and PCR positive subjects in different districts analyzed and summarized in Table 3.

4.2. Prevalence of Hepatitis C Virus Infection in Different Districts of Khyber Pakhtunkhwa

Out of the 390 collected samples, 35.90% (140) were found to be positive for HCV RNA, by PCR. Area wise distribution showed that, out of 185 ELISA positive samples collected from Swat, HCV RNA was detected in 67 (36.22%), out of 65 ELISA positive samples collected from Dir, HCV RNA was detected in 25 (38.46 %), out of 100 ELISA positive samples collected from Malakand HCV RNA was detected in 36 (36%), and out of 40 ELISA positive samples collected from Mardan HCV RNA was detected in 12 (30 %) subjects.

Table 1. Rate of Hepatitis C Virus Infection in Both Sexes of Khyber Pakhtunkhwa [a,b]

Gender	Total HCV Elisa Positive Samples	HCV RNA Detected, No. (%)
Male	200	81 (40.5)
Female	190	59 (31.05)
Total	390	140 (35.90)

[a] Abbreviations: HCV, Hepatitis C Virus; RNA, Ribo Nucleic Acid .
[b] P = 0.0103.

Table 2. Age Wise Distribution of Hepatitis C Virus Infection in Khyber Pakhtunkhwa [a, b]

Age, y	Total HCV Elisa Positive Samples	HCV RNA Detected, No. (%)
0-20	18	3 (16.67)
21-40	240	90 (37.5)
41-60	107	38 (35.51)
61	25	09 (36)
Total	390	140 (35.90)

[a] Abbreviations: HCV, Hepatitis C Virus; RNA, Ribo Nucleic Acid .
[b] P = 0.0000 .

Table 3. Rate of Hepatitis C Virus Infection in Different Districts of Khyber Pakhtunkhwa [a, b]

District	Total HCV Elisa Positive Samples	HCV RNA Detected, No. (%)
Swat	185	67 (36.22)
Dir	65	25 (38.46)
Malakand	100	36 (36)
Mardan	40	12 (30)
Total	390	140 (35.90)

[a] Abbreviations: HCV, Hepatitis C Virus; RNA, Ribo Nucleic Acid.
[b] P = 0.0000 (< 0.05) significant.

5. Discussion

Hepatitis refers to the inflammation of liver caused by multiple factors including many different viruses. Hepatitis A and E are usually transmitted by ingestion of contaminated food or water. Hepatitis B Virus (HBV) can be transmitted through contaminated blood, needles or unprotected sex with infected patients or by an infected mother to the child, while Hepatitis C Virus is most often transmitted through exposure to contaminated blood and needles, an infected mother to the child and rarely through unprotected sex with the patients. Hepatitis C Virus is spread worldwide. Countries which have reported high rates of infection are Egypt (15%), and Pakistan (4.8%). Around 130 to 170 million people of the world are infected with HCV and approximately three to four million individuals are being infected annually (32-35). In the last few years heavy monsoon rains which resulted in floods were considered as the worst calamity in the history of Pakistan (36), and they had a severe impact on population since floodwater destroyed most of the health care infrastructures in the worst-affected areas, leaving inhabitants especially vulnerable to water-borne diseases (37, 38). Different regions of Khyber Pakhtunkhwa, Upper Sindh, Southern Punjab and Balochistan were severely affected.

In the present study, the prevalence of HCV infection and its association with gender and age were analyzed in various areas (Mardan, Malakand and Dir and Swat districts) of Khyber Pakhtunkhwa. The most important finding of this work was that positivity rate of HCV is high (P = 0.0103 < 0.05) in male population compared with female population. Similar findings were also observed by Ali et al. (39) that male subjects were significantly more susceptible to HCV infection then female subjects and parallel results were also seen by another national study (40). But our results were contradict from Waqar et al. (41) that high HCV prevalence rate of HCV infection was seen in female patients as compared to male subjects. The reason could be that male population has more exposure to HCV causing risk factors. Another important finding of the current study was that HCV infection rate was significantly high in age group 21 to 40 and very low in age group of 0 to 20. These results are parallel with the findings of Ahmad et al. (42) that highest rate of incidence was seen in age group of ≤ 40 years and comparable findings were also observed by Ali et al. (43). But our results were in disagreement with findings of Muhammad et al. (44) that high HCV prevalence rate in Pakistan was seen in old age group people. So these results recommend that untimely detection of HCV may be due to the awareness of general public about HCV infection in this region of the globe. The third important result of the current research was that HCV infection was found to be significantly higher (38.4%) in Dir district compared to the other regions. In Swat, Malakand and

Mardan districts the observed prevalence of HCV was 36.2 %, 36% and 30%, respectively. The high prevalence of HCV infection in lower Dir district was due to low medical facilities and health centers, low awareness about the infectious disease, improper blood screening at the time of transfusion and etc. The prevalence of HCV reported from different regions of Khyber Pakhtunkhwa ranges from 4.1 to 36% (30, 31). To obtain a population based data about the prevalence of such infections, comprehensive research studies are needed. The current study was unable to identify the route of HCV transmission in the infected population selected in the current study. It was concluded that among all flood affected areas Dir district had the highest prevalence of HCV, the majority of the affected patients were among the age group 21 to 40 years, and male patients were more susceptible to this infection and the possible reason can be the high exposure of males to the risk factors of HCV infection.

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Authors' Contributions

Muhammad Waqar and Asad Ullah Khan collected epidemiological data and write manuscript. Amjad Ali, Muhammad Wasim, Muhammad Idrees, Zobia Ismail, Agha Asad Noor, Noorul Akbar,Shaista Bano, Muhammad Arif Khan and Rahim Ullah Khan analyzed and arranged the data. Noorul Akbar performed statistical analysis. Diagnosis was done at Genome Center for Molecular Based Diagnostics & Research (GCMBDR) Lahore.

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