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Immunochromatographic Technique for Seropositivity and PCR Based Molecular Diagnosis for Hepatis B and C Virus in a General Population of Mansehra Pakistan

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Abstract

Hepatitis B and C infections are known to be the major public health issue around the world. Our focus is to investigate the predominance of HBV and HCV infections in the general population of district Mansehra, Khyber Pakhtunkhwa, Pakistan. Blood samples were taken from 652 individuals and were shifted to Alkhedmat laboratory Mansehra and Department of Microbiology Hazara University Mansehra. Serum were isolated from blood samples and screen it by ICT device Kit (Accurate Diagnostics, Houston, Texas, USA). The ICT positive samples were then refined by PCR method. Total 652 individuals [males (368), females (284)] were screened for HBV and HCV infections in which total 37 (5.67%) [males (6.52%) and females (4.57%)] were found positive for HBsAg and 42 (6.44%) [males (7.33%) and females (5.28%)] were found positive for Anti HCV respectively. The ratio of these viral infections is more predominant as compare to other viral infections in Mansehra, Pakistan. The rate of HBV and HCV prevalence is increasing day by day in these areas. Communities with more than 5% HBV and HCV disease, mass vaccination and awareness programs should be undertaken as a matter of urgency.

Key words: HBV, HCV, ICT, PCR, Risk factors, Mansehra.

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1. INTRODUCTION

Hepatitis B and C are known to be the major public health issue in developing countries[1]. Viral Hepatitis is a condition in which liver attacking viruses (HAV, HBV, HCV, HDV, HEV, and HGV) can infect the liver and cause inflammation of liver. This leads to high morbidity and mortality in humans[2]. Patients with Acute hepatitis maintaining constant serum concentration of HBsAg will potentially become carriers and are vulnerable to chronic liver disease[3].

The degree of hepatitis B and C in Pakistan is serious treat[4]. Around the world different populations depending on their area have different prevalence rate of HBV and HCV ranging from 3-4% of Hepatitis B

and 5-6% of hepatitis C occurs. These studies show that in Pakistan approximately 12 million peoples are affected[5]. The prevalence of HBV transmission routes has been significantly varied across different geographical areas. According to statistical research, Sub-Saharan has been consider to have a high prevalence of HBV, whereas in Pakistan, India, Bangladesh, Sudan and Malaysia this virus is considered the main route of transmission, especially during infantile life and is called horizontal mode of transmission[6,7]. These viruses are sexually transmitted and intravenously in western Europe. Among acute and chronic liver diseases, HBV and HCV infections are clearly expressed. In Pakistan the hepatitis C virus is spreading mostly through some carriers which includes the use of contaminated surgical instruments and needles in medical practices and the transfusion of unsafe blood[8,9]. Reason for this issue may be poor economic status, unproper health facilities and unawareness in public about the spreading and transmission of these viral diseases[10].

The major aim of this study was to highlight the predominance of Hepatitis B and C virus infections in the general population of district Mansehra and to find the causative agent through which this deadly disease is spreading.

2. MATERIALS AND METHODS

2.1 Studied Area

This cross-sectional study was done in district Mansehra Hazara division in the duration of September 2018. All the blood samples were collected from male and female volunteers in Mansehra city and were shifted to Al-Khidmat laboratory, near King Abdullah hospital Mansehra City and Department of Microbiology Hazara University Mansehra for further processing.

2.2 Samples collection

Total 652 individual were studied. Under the aseptic conditions, blood samples were collected from each volunteer in a vacutainer tube and labelled with personal information. Blood samples were then centrifuged, and the serum was tested for HBsAg and anti-HCV antibodies. Remaining serum of positive samples kept at -40°C for the PCR analysis.

2.3 Virology Screening

The Immunochromatographic Technique, HBsAg (ICT) 2.0 Kit (Accurate Diagnostics, Houston, Texas, USA) was used to investigate the HBsAg. The reactive samples were then refined By PCR method. Similarly, for anti-HCV antibodies detection, HCV version 3.0 ICT Kit (Accurate Diagnostics) was used. Serologically positive HCV samples were further subjected to PCR for further confirmation.

2.4 Nucleic acid extraction of HBV and HCV

The extraction of HBV DNA and HCV RNA for serum samples were done through Ana-gen DNA/ RNA extraction kits (LOT No. 69902, Duluth, Georgia, USA), according to the manufacturer's protocol.

2.5 PCR for hepatitis B virus

First round of PCR

The extracted DNA from HBsAg positive samples were then subjected to PCR for surface gene amplification. The PCR was processed in two rounds due to low DNA concentration. The primers for first round of PCR (forward and reversed) is Different than second round of PCR (forward and reversed)[4]. In the First round of PCR forward primer 5' CATCCTGCTATGCCTCATCT 3' and reversed primer 5' CGAACCACTGAACAATGGCACT 3' was used[4]. Total 20.0 µl PCR mixture was prepared for first round of PCR using $MgCl_2$ (1.5 µl), dNTPs (1.0 µl), Taq buffer (2.0 µl), Taq polymerase (0.2 µl), Forward primer (1.0 µl), Reverse primer (1.0 µl), DNA Template (10.0 µl) and DDH2O (3.3 µl). The samples in the first round of PCR were incubated in the Thermo cycler (ABI PCR system 2700) at 94°C for five minutes and onward in 2nd step of first round the samples were incubated by 35 cycles in which each cycles was adjusted for denaturation at 94°C for 30 sec, annealing at 53°C for 40 sec, extension at 72°C for 30 sec and final extension at the last stage for 5 minutes at 72°C.

Second round of PCR

In the second round of amplification Forward primer 5' GGTATGTTGCCGTTGTCCTCT 3' and reverse primer 5' GGCACTAGTAAACTGAGCGCCA 3' was used[4]. Total 20.0 µl PCR mixture was prepared for second round of PCR using $MgCl_2$ (1.5 µl), dNTPs (1.0 µl), Taq buffer (2.0 µl), Taq polymerase (0.2 µl), Forward primer (1.0 µl), Reverse primer (1.0 µl), DNA Template (4.0 µl) and DDH2O (9.3 µl). The samples in the second round of PCR were incubated in the Thermo cycler (ABI PCR system 2700) at 94°C for five minutes and onward in 2nd step of second round the samples were incubated by 35 cycles in which each cycles was adjusted for denaturation at 94°C for 30 sec, annealing at 53°C for 40 sec, extension at 72°C for 30 sec and final extension at the last stage for 5 minutes at 72°C.

2.6 RT-PCR for HCV RNA (cDNA synthesis)

The extracted HCV RNA was subjected to RT-PCR for both Complimentary DNA synthesis and amplification of the target sequence performed in single tube by using the one-step RT-PCR kit (Puregene, Minnesota, USA) according to the manufacturer's protocols. Primers were designed that bind specifically to the 5'-U region in the HCV genome which are greatly conserved. the following primers sequences were used: outer sense primer (5'-CCCTGTGAGGAACTWCTGTCTTCACGC-3'); antisense outer primer (5'-GGTGCACGGTCTACGAGACCT-3'); inner sense primer (5'-TCTAGCCATGGCGTTAG TRYGAGT GT-3'); and inner antisense primer (5'-CACTCGCAAGC ACCCTATCAGGCAGT-3',W=A or T, R=A or G, Y=T or C)[25]. The reactions were carried out in 25µl volumes using 10 µl RNA in the presence of 0.6µmol/l of each HCV outer primer, 400µmol/l dNTP and five unit's RNase inhibitor. The PCR thermocycler (Eppendorf, Germany) was adjusted as: one cycle at 50°C for 30 min, one cycle at 95°C for 15 min followed by 40 cycles at 95°C for one min, 55°C for 1 min and 72°C for 1 min. The reactions cycles were complete at 72°C for 10 min.

2.7 Nested PCR for HCV cDNA

The cDNA concentration was less that's why Nested PCR was performed to improve the detection capacity of the PCR. The second PCR reactions were carried out in 25 µl volumes using 5 µl DNA templates from first PCR with 1× PCR master mix (Promega, USA) and 0.4 µmol/l of HCV inner primers. The PCR thermocycler (Eppendorf, Germany) was adjusted for cycling conditions as: one cycle for 2min at 94°C, onward 35 cycles of incubation for 30 sec at 94° C, 45 sec at 58°C, and 1 min at 72°C, and the final extension was done for 7 min at 72°C.

2.8 Gel electrophoreses

All the PCR products was mixed with loading dye (3µl) and then analysed on 1% agarose gel with 10 Kb DNA ladder. The agarose is prepared in 1x TAE (Tris-acitate-EDTA) buffers, which was stained with ethidium bromide and was evaluated on Ultraviolet transilluminator. The size of PCR products was compared with 10 Kb DNA ladder (Fermentas Life Sciences).

2.9 Statistical analysis

All the data were Statistically analyzed, Summarized and Calculated by using MS Excel and SPSS version 10.0 (SPSS Inc., Armonk, New York, USA).

3. RESULTS AND DISCUSSIONS

3.1 Prevalence of Hepatitis B Virus

Total 652 individuals [males 368 and females 284] were screened for HBV infections in which 37 (5.67%) individuals including 24 (6.52%) males and 13 (4.57%) females were found reactive to HBsAg Shown in Table-1. These positive sample were then subjected to PCR method to analyse the DNA in which 16 (43.24%) individual were found reactive including 11 (45.83%) males and 5 (38.45%) were females. Gender wise prevalence of HBV indicates that males are more affected as compared with female individuals.

Table 1. Gender wise Prevalence of HBV

Gender	Total screened samples n= %	ICT +ve samples n=%	PCR +ve samples n=%
Male	368 (56.44%)	24 (6.52%)	11 (45.83%)
Female	284 (43.56%)	13 (4.57%)	5 (38.45%)
Total	652 (100%)	37 (5.67%)	16 (43.24%)

Recent updates show that the HBV infections are going to be the most highlighted health issue globally including high predominance in south Asian countries[11]. Hepatitis B virus is blood born pathogen and survive mostly in blood to increase their progeny. In Pakistan about 9 million peoples were infected by HBV and this burden is increasing day by day due to transmission from person to person[12]. Our study reveals that 5.67% people were infected by HBV in Mansehra region which includes high ratio of males than females. According to literature review about 8% prevalence was found in Rahim Yar Khan which was greater than prevalence found in Gujranwala (5%), Pak Pattan (5.32%), Dilpalpur (3%), Lahore (2.3%) and Liaqatpur (6.99%) [13]. Similarly, the prevalence of HBV detected in Islamabad and Karachi were 5.3% and 6.5% respectively[14,15] while in Faisalabad prevalence was noted 4.5%[16].

Our data shows that the prevalence in Mansehra is greater than Gujranwala, Dilpalpur, Lahore Islamabad, and Faisalabad. Alam et al.[17] reported in Nowshera region was 4.9% which is relatively less than our prevalence Shown in Fig. 1. The prevalence found in male individual are relatively higher as compare to female individuals, this is due to the high accessibility of males with high risk factors and areas like hospitals and barbers etc. In female individuals the risk of HBV transmitting is due to unsafe blood transfusion during pregnancy or labour period when patient loss enough blood. In the spreading of HBV virus some of unqualified or untrained technician's perceptions play major role by using contaminated needles, surgical instruments and drug abuses which raises HBV extremely worst. Mostly people's share their shave blades and other equipment's in hostels and barbers' shops may leads Hepatitis transmittance. It is suggested that every patient must be screened for HBV before Surgical practices. The hepatitis Patient must be facilitated separately by providing separate operation rooms and sterile surgical instruments.

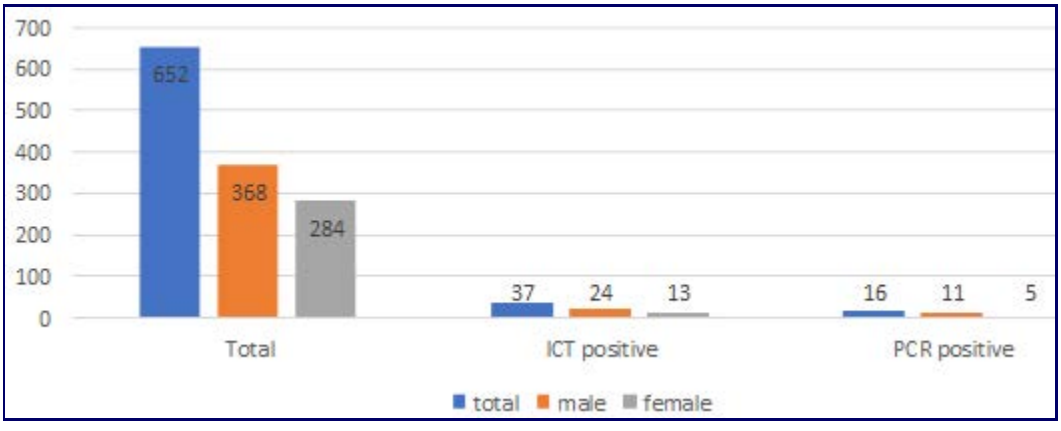

Fig. 1. shows gender wise prevalence of hepatitis B virus in district Mansehra.

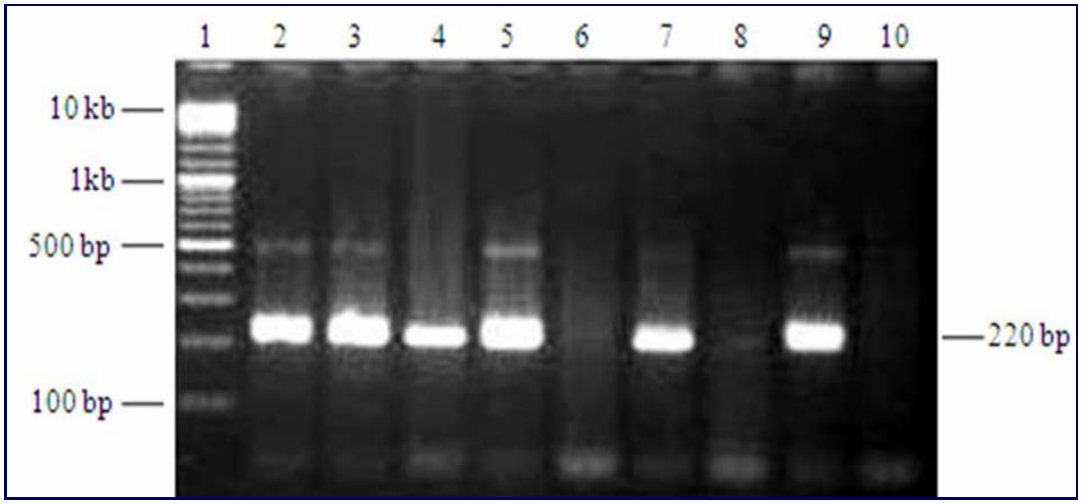

Fig. 2. shows HBV detection via PCR. It was used with 1% agarose gel. The marker having size 10kb used in Line 1; positive results appear in line 2, 3, 4, 5, 7, and 9; negative results appears in line 6, 8, and 10.

3.2 Prevalence of Hepatitis C Virus

The result indicates that total 652 individuals [368 males and 284 females] were screened for HCV infections in which 42 (6.44%) individuals including 27 (7.33%) males and 15 (5.28%) females were found reactive to anti HCV. the positive samples were then refined by PCR method in which 21 (50%) individuals including 15 (55.55%) males and 6 (40%) females were positive as Shown in Table 2. Like HBV infection the HCV is also more prevalent in the males as compared to females. This is because male individuals are more expose to risk factors.

Table 2. Prevalence of HCV

Gender	Total screened samples n=%	ICT +ve samples n=%	PCR +ve samples n=%
Male	368 (56.44%)	27 (7.33%)	15 (55.55%)
Female	284 (43.56%)	15 (5.28%)	06 (40%)
Total	652 (100%)	42 (6.44%)	21 (50%)

According to literature review the prevalence of HCV previously reported in overall Pakistan was 4.7%[18], 4.95%[19], and then 4.87%[20]. One of the studies estimated that in Pakistan about 6.8% adults are infected by HCV infections. Our findings reveal that in Mansehra region the predominance of HCV is 6.4% which is higher than the overall predominance in Pakistan. A study conducted in Mardan reported that 4.69% peoples are infected from HCV[21] which means Mansehra peoples are more affected than Mardan peoples, whereas in Faisalabad prevalence was estimated round about 16% which is very higher than other regions in Pakistan[22].

Worldwide the prevalence of HCV is also in its peak but one of the studies conducted in Italy reported that 14.4% peoples were infected by HCV in Italy[23]. The prevalence is raising day by day due to the human's negative activities.

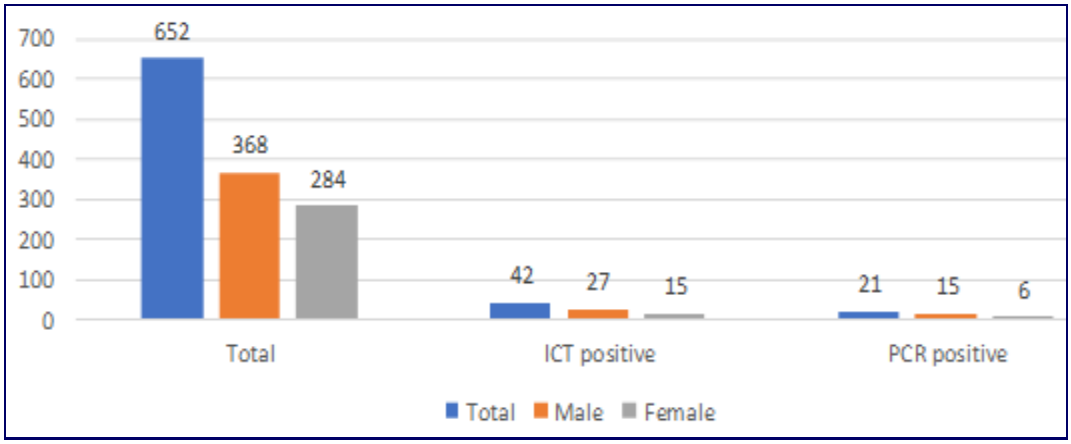

Fig. 3. shows gender wise prevalence of hepatitis C virus in district Mansehra.

The HCV infected swat population was shown to be in treatment status (58.4%) patients receiving treatment properly. In these Interferon therapy non-responders were (4.9%), while about (5.94%) patients never getting proper treatment. among treating patients' resistance to interferon and ribavirin combination therapy was found in (4.86%). Current available therapies to treat HCV infection have adverse effects and lower virologic response rates[23]. Sustained virologic responses in Pakistani population (6 months versus normal interferon and ribazole) was noted in 72.25% and 27.7% respectively[24]. HCV patients who had previously received interferon plus ribavirin standard therapy and were either non-responders or initially responding to the therapy (end of therapy response) but subsequently recurred. During its replication cycle, HCV mutants vary rapidly, allowing very common drug resistance mutations to emerge. In a population of viruses already present, there is a rapid emergence of new strains or subspecies, enabling the new or resistance virus to become the leading strain. therefore, in order to properly research the genetic positive of HBV and HCV of these newly emerging strains clinical laboratories may need to modify their genetic assays because a specific genetic assay may not be found in previously developed assays.

4. CONCLUSIONS

The prevalence of Hepatitis B and C virus is raising day by day. To reduce the spreading and transmission of this deadly viruses, peoples must aware of the causative agents and risk factors of these infections. For the already infected patient's novel treatment strategies must be identified to overcome the disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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