

# An evidence of high prevalence of Hepatitis C virus in Faisalabad, Pakistan

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## ABSTRACT

**Objective:** To see the prevalence of Hepatitis C virus (HCV) and its genotypes in general population of the locality and to evaluate the importance of reverse transcriptase-polymerase chain reaction (RT-PCR) test over HCV antibody test in early diagnosis of HCV infection.

**Methods:** The study took place at the National Institute for Biotechnology and Genetic Engineering (NIBGE) and we collected the samples from HCV screening camps organized by Allah Rakhi Trust Hospital Faisalabad and from blood donors visiting to Allied Hospital Faisalabad. Blood samples from 300 subjects (77% males and 23% females) with a mean average age of  $32 \pm 20$  years were randomly collected, and analyzed after separating the plasma by serological testing as well as molecular methods during the period from January 2004 to December 2004. Genotyping of HCV positive samples were performed by restriction fragment length polymorphism (RFLP) analysis of the PCR products.

**Results:** The results indicated that 48 (16%) cases were positive for anti-HCV antibody and 14 (4.7%) were on the borderline. Alanine aminotransferase (ALT) level indicated a wide range of activity from 5.0U/L to 128U/L. On the average, 79.4U/L activity was found. By nested PCR, 84 (28%) samples were positive for HCV-RNA. The results of genotyping have indicated that 8 (9.5%) samples were of genotype-1 while only 2 (2.4%) samples were of HCV genotype-2. Hepatitis C virus genotype-3 was found to be major prevalent genotype as it was present in 68 (81%) samples. Six (7.1%) samples could not be identified by this method thus remained un-typed.

**Conclusion:** Hepatitis C virus-RNA detection by molecular method was found to be more sensitive for early diagnosis of HCV infection and relatively higher incidence of HCV infection with genotype-3 was found in general population of the area.

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Hepatitis C virus (HCV) was found to be a major cause of non-A non-B (NANB) hepatitis in the world.<sup>1</sup> It is responsible for parenterally transmitted, acute and chronic hepatitis. The persistent infection (>50%) due to HCV usually results in chronic active hepatitis that may lead to liver cirrhosis and hepato-cellular carcinoma.<sup>2</sup> In some areas of the world, the prevalence of HCV remains very high suggesting that some, yet unknown; vector might be involved in its transmission in those areas. Approximately 3% of the world's population is chronically infected with HCV<sup>3</sup> and constitute a medical threat, underscoring the urgent need for anti-HCV vaccines and antiviral agents.<sup>4</sup> Many advances have been made during the last 10 years including characterization of HCV with its genetic diversity, development of the third generation antibody diagnostic test in most clinical, better understanding of epidemiology and its treatment with alpha-interferon.<sup>5</sup> At present, anti-HCV ELISA test is the only diagnostic test in most clinical laboratories of the world, which is not so valuable especially for its early diagnosis or for the past or resolved cases. The present study was undertaken to detect HCV infection in volunteer blood donors as well as from the general population of Faisalabad, being the biggest industrial and as such polluted city of the country with ongoing malpractice among the medical community in treating the patients. In this study, various serological and molecular techniques such as in-house reverse transcriptase-polymerase chain reaction (RT-PCR) methods were applied to see HCV prevalence in the area and their comparative study was carried out for early diagnosis of the disease. The genotyping of HCV which is considered important in its management was also carried out.

**Methods.** The study was carried out at the National Institute for Biotechnology and Genetic Engineering (NIBGE). For this purpose, blood samples from 300 subjects (232 males and 68 females) with an average age of 32±20 years were collected at random after filling the detailed verbal history Performa of each subject. These samples were taken from blood donors visiting to the Blood Bank, Allied Hospital Faisalabad and from HCV screening camps in collaboration with Allah Rakhi Trust Hospital Faisalabad in the time span of 6 months from January 2004 to June 2004. The samples were analyzed by serological test as well as molecular methods after separating plasma during the next 6 months (July 2004 to December 2004). The detection of antibodies of HCV in the samples was performed by one step cassette style anti-HCV device as per instructions from the manufacturer. The presence or absence of anti-HCV antibodies in the samples was determined by appearance of specific colored line on the detection device.

**Alanine aminotransferase (ALT) level.** The biological marker of the liver inflammation and necrosis, was determined by using Liqui-UV-Kit, (Germany) according to the instructions from the manufacturer.

**Reverse transcriptase-polymerase chain reaction.**  
**a) viral RNA extraction and cDNA synthesis:** All the reagents for RNA extraction were made in 0.1 % Diethyl Pyrocarbonate (DEPC) treated water. Ribonucleic acid from the plasma was extracted according to the procedure.<sup>6</sup> Briefly, 100µl of plasma or serum was incubated with Guanidinium isothiocyanate lysis solution for 20-30 minutes and the RNA was precipitated with isopropanol by centrifugation, washed once with 70% ethanol and re-suspended in 10µl DEPC treated water after drying the pellet. Complementary DNA (cDNA) was synthesized from the RNA extracted by using reverse transcriptase enzyme following the protocol<sup>7</sup> with some modifications. Briefly, 20 µl reaction mixture containing 10 µl RNA sample, 1X first strand buffer, 100 µmol dNTPs, 20pmol of the anti-sense primer (AS1: 5'-GTGCACGGTCTACGAGACCT-3', positions -1 to -21), 2 units of ribonuclease inhibitor and 50U of Reverse transcriptase enzyme was incubated at 42°C for one hour.

**b) Regular polymerase chain reaction:** In-house regular PCR was performed with the outer primers (AS1 and S1) complimentary to the 5' UTR of the HCV genome. Polymerase chain reaction was performed with a total reaction volume of 25 µl containing 05 µl of the synthesized cDNA of the samples as template and with final concentration of each, 100 µmol dNTPs, 1X PCR buffer, 25 pmol of the both primers, sense (S1: 5'-GCCATGGCGTTAGTATGAGT-3', position; -259 to 240) and anti-sense (AS1: 5'-GTGCACGGTCTACGAGACCT-3', position;-1 to

-21) and 5 units of Taq DNA polymerase. The reaction volume was adjusted with sterile distilled water. For PCR amplification, first cycle of the temperature profile 94°C for 3 minute was used. It was followed by 30 cycles with the temperature profile 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and finally a cycle at 72°C for 3 minutes for fragments extension.

**c) Nested polymerase chain reaction:** For nested PCR, 1-2µl of the regular PCR product was used as template while internal primers, both sense (S2: 5'-GTGCAGCCTCCAGGACCC-3, position -237 to -220) and anti-sense (AS2: 5'-CCGTGAGCGTTCGTGGGATA-3', position;-27 to -46) were employed.<sup>7</sup> Other conditions were the same as in case of the regular PCR. The amplified PCR products of nested PCR were run on 1.5% agarose gel following<sup>8</sup> and visualized under ultraviolet light.

**Hepatitis C virus genotyping:** HCV genotypes were determined by RFLP analysis of the nested polymerase chain reaction (N-PCR) products of the HCV positive samples, as described<sup>9</sup> with minor modifications. To differentiate HCV into its major genotypes, positive nested-PCR products were restricted by 3 sets of restriction enzymes; (i) Hae111 and Rsa1, (ii) Scrfl and Hinf1, and (iii) Mva1 and Hinf1. The restricted PCR products were separated on 4% agarose gel in 1X TBE solution. The nomenclature proposed by McOmish<sup>10</sup> and Simmonds,<sup>11</sup> was used for the HCV genotypes identification based on PCR-RFLP analysis with some minor modifications.

**Results. General criteria:** Three hundred blood samples collected from blood donors and from HCV screening camp were 232 (77.3%) males and 68 (28.7%) females, with an average age of 32 years (Table 1). Almost all these subjects were looking healthy and energetic with no signs of any disease, weakness, nausea or pyrexia except for a few cases with the history of jaundice that were 12 (4%) in number. There was no case of intravenous drug abuse or hemodialysis and only 6 persons with the history of blood transfusion. All the samples were screened for HCV by one step cassette style anti-HCV device. The results obtained by this test indicated that 62 (20.6%) cases were positive for anti-HCV antibodies (anti-HCV). All other samples were found negative for anti-HCV antibody test.

The results of Alanine aminotransferase (ALT), whose level disturbed during liver infection indicated a wide range of activity from 5.0U/L to 128U/L, when samples were spectrometrically assayed at 25°C. The average of 79.2U/L activity was found in all the 300 samples tested (Table 1 & Figure 2). The normal range of the kit was 0-40U/L. Regular PCR of all the samples was carried out using external primers, while nested PCR was carried

**Table 1** - Interrelation of various age groups with hepatitis C virus-ribonucleic acid (HCV-RNA) detection and alanine aminotransferase (ALT) level in samples from general population.

Age groups (years)	Gender M/F	No. of total subjects (%)	No. of HCV-RNA positive subjects (%)	Mean ALT level U/L
10-20	10/4	14 (4.7)	4 (1.3)	43 ± 36
21-30	94/32	126 (42.0)	31 (10.3)	64 ± 54
31-40	102/21	123 (41.0)	35 (11.7)	81 ± 76
41-50	15/6	21 (7.0)	10 (3.3)	99 ± 45
51-60	11/5	16 (5.3)	4 (1.3)	110 ± 95
Total	232/68	300 (100)	84 (28.0)	79.4 ± 61.2

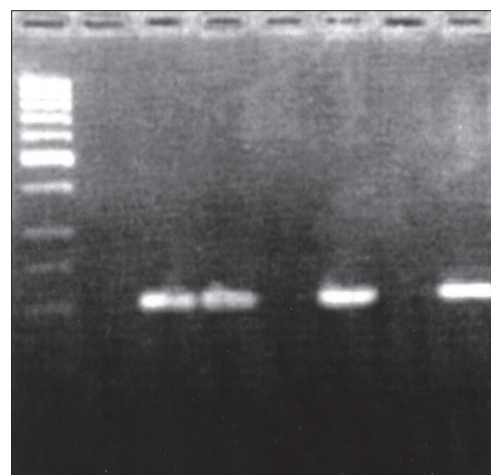
**Table 2** - Prevalence of hepatitis C virus (HCV) genotypes in samples from general population.

Genotypes	n	(%)
Genotype-1	8	(9.5)
Genotype-2	2	(2.4)
Genotype-3	68	(81.0)
Un-typed	6	(7.1)
<b>Total</b>	<b>84</b>	<b>(100)</b>

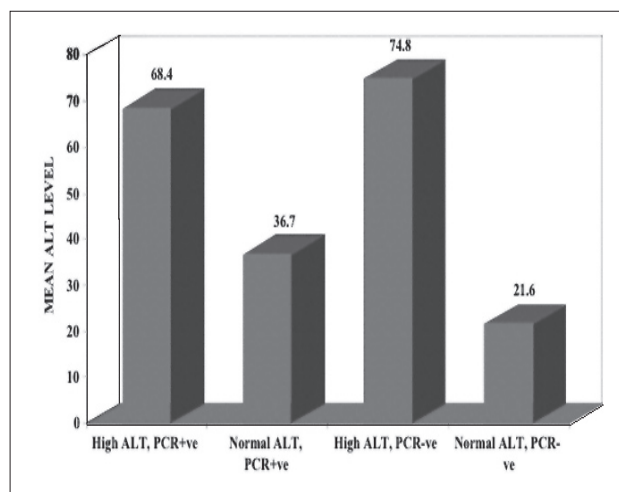
out using internal set of primers. Both negative and positive samples were used as control and 50 bp DNA ladder (Fermentas) as DNA marker. Polymerase chain reaction products of N-PCR indicated amplification in several samples when checked on agarose gel (Figure 1). By N-PCR, 84 (28%) subjects were found positive for HCV-RNA, while 216 (72%) were found negative for HCV-RNA. Among these subject, 53 (17.7%) were both PCR and anti-HCV positive while 31 (10.33%) were only PCR positive, however these were anti-HCV negative. Similarly, 9 (3%) samples were found anti-HCV positive although these were PCR negative.

The genotyping of HCV-RNA positive samples was carried out by PCR-RFLP analysis. The results of genotyping are summarized in Table 2 indicated that HCV genotype-3 was found in 68 (81%) of the PCR positive samples while 2 (2.4%) samples were of genotype-2 and 8 (9.5%) samples were of genotype-3. Six samples (7.1%) could not be identified by this method.

**Discussion.** Hepatitis C virus is a major health problem all over the world and its prevalence varies widely among the different regions of the world. During this study, 300 samples from subjects of various age groups were taken from general population in order to get an idea about the prevalence/incidence of HCV



**Figure 1** - Analysis of samples for hepatitis C virus detection by agarose gel electrophoresis after reverse transcriptase-polymerase chain reaction (RT-PCR). Lane 1: DNA size marker, Lane 2: negative control, Lane 3: positive control, Lanes 4-8: specific RT-PCR products of HCV positive and HCV negative samples.



**Figure 2** - A comparison of plasma alanine aminotransferase (ALT) level versus reverse transcriptase-polymerase chain reaction (RT-PCR) in samples from general population. Subjects (%): High ALT, PCR +ve = 20.0, Normal ALT, PCR +ve = 08.0, High ALT, PCR -ve = 7.4, Normal ALT, PCR -ve = 64.6

infection in the area. It was found that 62 (20.6%) subjects were positive by anti-HCV screening while in case of RT-PCR, 84 (28%) subjects were positive. The results indicated that in RT-PCR, even very recent cases of HCV infection can be detected that are not yet sero-converted. These findings emphasized the superiority of molecular testing over routine serological testing for early diagnosis of HCV infection. From comparative data of anti-HCV test with RT-PCR, it was noted that in 13% cases, discrepant results were found between RT-PCR and anti-HCV test.

Anti-HCV screening test or anti-HCV-ELISA test for the detection of anti-HCV antibodies (anti-HCV) have been evolved since 1990 and third or even fourth-generation of such assays is now available that detects anti-HCV in almost 95% cases of HCV infection<sup>12</sup> and is the only screening test in most clinical laboratories of the world, which is still not so specific and sensitive especially, for early diagnosis of HCV infection and also in immune-suppressed patients. Also, these assays do not distinguish between onset of acute illness and sero-conversion due to 2-12 months of window period of sero-negativity after acute infection and occasionally gives false antibody reaction.<sup>13</sup>

Anti-HCV antibody test data of this study was concordant with another report in which 18.4% of healthy donors from Egyptian peoples residing in Saudi Arabia were found to be positive by ELISA,<sup>14</sup> hence indicating hot areas of high prevalence of HCV infection in the general population but the data was discordant to another study performed in Italian population where overall confirmed anti-HCV prevalence was only 2.4%.<sup>15</sup>

Reverse transcriptase-PCR results of these subjects indicated even more alarming situation as higher number (28%) of subjects were found to be HCV-RNA positive as compared to anti-HCV (20.6%). One possible explanation of such high prevalence of HCV detected by RT-PCR might be that these individuals were very recently infected by HCV and specific antibodies were not yet formed in enough quantity to be detectable by the HCV screening device or they were asymptomatic carriers of HCV-RNA, as has been reported earlier.<sup>16</sup> The cases, which were negative by anti-HCV antibody test but positive with RT-PCR, also confirmed the previous findings that viral sequence could be found in individual whose anti-HCV test was negative.<sup>17</sup> In ELISA test, anti-HCV antibodies are detected but the appearance of antibodies in the blood after the onset of hepatitis can be delayed from 3-52 weeks.<sup>18</sup> It was also noted during this study that most of the individuals that were HCV positive either serologically or RT-PCR, have a higher level of ALT. There was generally good co-relation between the positive samples and high ALT level. But,

some discrepancies were also noted where ALT level was higher even in the absence of HCV detection or vice versa. Some individuals had normal ALT level even in the presence of HCV-RNA, which confirmed the previous findings that HCV associated infected persons may be characterized by alternate periods of high ALT activity and quiescent periods in which ALT level is normal.<sup>19</sup> Conflicting data concerning the presence of histological confirmed liver damage indicated that invariable association between the detectable HCV viremia and liver injury in the absence of raised ALT level values exists.<sup>20</sup> The reasons of high incidence of HCV in the general population of this highly populated area consisting of less educated people might be (i) lack of awareness about risk factors involved in its transmission/spread (ii) malpractice among medical community such as reuse of syringes, and not using properly sterilized medical instruments especially by Dentists and (iii) reuse of contaminated razors by the Barbers and so forth. Yet another important reason for higher rate of HCV carriers might be that presently, facility for HCV screening in most blood transfusion centers of the country are not up to the standard, thus a major source of transmission of HCV infection. To draw a clear-cut picture about the prevalence of HCV infection in general population of Pakistan, a number of screening programs of HCV infection detection are needed. Some unknown vector yet to be identified may also be involved in its spread to the healthy population. Being a big industrial center of the country, general hygienic conditions and environmental pollution are also major problems of the locality that might also contribute its infection. Mosquito might also be a strong candidate in this respect and its involvement in HCV spread/transmission is needed to be focused. After confirmation of HCV infection with RT-PCR, it was important to know the HCV genotypes prevalent in the area. The results indicated that HCV type-3 was the most prevalent genotype in the area as shown in **Table 2**. There is a great diversity in HCV genotypes around the world and geographical localization of some genotypes are associated with special routes of infection.<sup>21,22</sup> Genotyping results of this study were not in accordance with some early findings in which genotype-1a and 1b (G1) were found the prevalent genotypes in some parts of the world including Canada, USA, Iran and Tunisia and so forth.<sup>23-29</sup> Similarly, these findings were also contradicted to other reports in which HCV genotype-2 (G2) was found more common than HCV-1 in some other parts of the world such as Indonesia, China, Argentina and Italy.<sup>7,30-33</sup> Prevalent HCV genotypes in the area showed very close concordance with another study carried out in India where a high prevalence of genotype 3 (>76%) and a very low prevalence of G2



(<2%), was documented in 153 samples representing different regions of the country.<sup>34</sup> Few data about the HCV infection/incidence and particularly about its genotypes common in the specified area is available. Thus, the knowledge of HCV genotypes present in Pakistan which is just like India might be helpful in its management or treatment. As HCV-3 was the major genotype prevalent in the area which it possessed some plus points also as reported earlier that among HCV genotypes, HCV-1b was associated with more severe liver disease<sup>35</sup> and appeared to be less responsive to interferon therapy than HCV-2 and HCV-3.<sup>7, 36-38</sup>

In conclusions, the findings of the present study are unique that the area under investigation is lying among the area of the world where the HCV infection is higher. Preventative measures might be sufficient to combat HCV transmission in an environment where the economic pressures are too great. The knowledge about genotyping may therefore be important in development of community-based vaccine in future. It is also suggested that the screening of blood for transfusion should be made compulsory, making blood transfusion safer for the recipients and avoiding of risk factors involved in its transmission to the healthy persons. It was further found that molecular diagnosis of HCV by RT-PCR is more superior, specific and sensitive method than serological testing especially for its early diagnosis and also for past resolved cases.

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