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Prevalence and Genotypic Analysis of Hepatitis C Virus Isolated from Liver Center, Faisalabad, Pakistan

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ABSTRACT

Hepatitis C is a serious concern to public health sector, particularly in developing countries like Pakistan. HCV is the significant cause of morbidity and mortality globally due to the lack of HCV vaccine. HCV genotypes are helpful for analyzing disease severity and start of therapy. Therefore, this study was designed to determine the genotyping of clinically isolated HCV from a Liver Center Faisalabad, Pakistan. A total of 3200 clinical samples were screened for the presence of anti-HCV antibodies by ICT method. Among positive samples, confirmation was carried out by RT-PCR and clustered into four groups (acute, chronic, cirrhotic and hepatocellular carcinoma) on the basis of clinical reports and the patient's history. HCV genotypes were determined by RFLP using amplified products and modified multiplex RT-PCR. Out of 3200 clinical samples, 5.37% (n=172) samples were HCV positive by ICT method. Among these, 96 (55.8%) were confirmed for HCV positive patients. Multiplex RT-PCR data showed that 75 (78%) patients had single HCV genotype infection (3a); three with mixed HCV infection (3a/2a, 3a/3b and 3a/1a) while 18 samples had no HCV signal. The current study demonstrated the high prevalence of HCV in our locality with the most prevalent HCV genotype 3a.

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Authors' Contribution
MS collected samples and performed practical work. AM and NA presented the idea and supervised the research work. MS and MUQ analyzed the data and wrote the manuscript. NS and BA cirtically reviewed the manuscript.

Key words
HCV, HCV Genotypes, Hepatocellular carcinoma, Multiplex RT-PCR

INTRODUCTION

Hepatitis C virus (HCV) is a serious public health concern globally, particularly in developing countries like Pakistan and the leading cause of high morbidity and mortality worldwide. According to the World Health Organization (WHO), about 71 million people are infected with HCV globally. Among these, almost 400,000 people die per year, primarily due to liver cirrhosis and hepatocellular carcinoma (WHO, 2017) and 30% of HCV positive patients lead to liver cirrhosis worldwide (Nazir *et al*., 2017; Aslam and Aslam, 2001). HCV is a positive sense single stranded RNA virus that was discovered primarily in 1989 belonging to a virus family *Flaviviridae* (Butt *et al*., 2010; Idrees *et al*., 2008; Choo *et al*., 1991). HCV genome is approximately 9.6 kb in length with single open reading frame and encodes a polypeptide of 3000 amino acids (Idrees and Riazuddin, 2008).

The two virological outcomes of natural HCV infection are acute infection with subsequent clearance of virus and virus persistence which leads to chronic infection. Only about 30% of HCV infected patients naturally clear HCV, while majority of them remain persistently infected. Active replication of HCV occurs in these patients for decades, leading to chronic hepatitis progressing into fibrosis, then cirrhosis and ultimately resulting into hepatocellular carcinoma (HCC) and liver failure. Chronic HCV infection with associated pathologies is responsible for about 250,000 deaths per year worldwide (Kocabayoglu and Friedman, 2013).

In Pakistan, HCV infection is very common and no comprehensive nation-wide data available about HCV prevalence. However, a few studies are available that have been conducted in different districts, cities and towns for the identification of HCV genotypes in chronic HCV infected patients circulating in that particular areas (Ali *et al*., 2010; Inamullah *et al*., 2011; Idrees, 2001). According to WHO estimates, Pakistan has the second highest prevalence of HCV worldwide. About 5% of the total Pakistani population is infected with HCV and the highest prevalence of HCV (7%) was observed in the

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Punjab province (Umer and Iqbal, 2016) while 10.4% HCV preva-lence was observed in District Shangla, Khyber Pakhtunkhwa province (Khan *et al*., 2018). Two Pakistani studies also documented the prevalence of HCV in Faisalabad (28%), Gujranwala (23%) and Lahore (16%) (Ahmad *et al*., 2007; Aslam and Aslam, 2001).

HCV prevalence and genotypes are variable in different regions of the world and among different groups of the community. HCV genotypes have played a vital role in the therapeutic outcome of HCV infected patients. There are almost six major HCV genotypes (1 to 6) have been identified so far which are further classified into many subtypes including 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c, 4a, 4b, 5a, 6a (Nazir *et al*., 2017; Nouroz *et al*., 2015; Idrees and Riazuddin, 2008). The global prevalence of anti-HCV was estimated to be 1.6%, corresponding to 115 million past viremic infections. The majority of these infections (104 million), were among adults (>15 years old) with an anti-HCV infection rate of 2.0%. Moreover, the viremic (RNA positive) prevalence was forecasted to be 1.1% that corresponds to 80 million viremic infections (Gower *et al*., 2014). Genotype 1 was most common i.e. 46% of all infections, followed by genotypes 3 (22%), and genotypes 2 and 4 (13% each) worldwide. Subtype 1b accounted for 22% of all infections at the global level. However, significant variations were observed across regions with genotype 1 dominating in Australasia, Europe, Latin America and North America (53-71% of all cases) and G3 accounting for 40% of all infections in Asia. Genotype 4 was most common (71%) in North Africa and the Middle East, but when Egypt was excluded, it accounted for 34% while genotype 1 accounted for 46% of infections across the same region (Gower *et al*., 2014).

In Pakistan, high prevalence of HCV genotypes 3 has been reported in different cities such as in Rawalpindi (96%) and Islamabad (96%) (Afzal, 2017), Quetta (50%) (Afridi *et al*., 2009) and Faisalabad (81%) (Ahmad *et al*., 2007). The current study is also an extension of our previous study in Faisalabad in order to maximize the chances of successful treatment outcome for each individual patient, rendering HCV genotyping assays important and useful tools to optimize treatment type, duration and dose. Faisalabad city is the 3rd largest populated and industrial city of Pakistan. Therefore, this study was designed to determine the dissemination of HCV genotypes from clinically isolated HCV in Faisalabad.

MATERIALS AND METHODS

Ethical consideration

Before starting the research project, ethical approval was obtained from the ethical review committee, Government College University, Faisalabad.

Sample collection

A total of 3200 clinical blood samples were collected from HCV suspected patients from a liver center, Faisalabad, Pakistan from January, 2018 to June, 2018. A brief history of each suspected patient was taken from each patient including, demographic characteristic, age, gender, occupation, surgery, visit to the dentist and/ or barber, drug user district, and estimated time of infection along with complete address and phone number of the patients. Moreover, a written informed consent was also taken from each patient.

Screening of HCV

A total of 5mL venous blood samples were drawn from each individual in the yellow top vacutainer tube (BD®, USA) aseptically. Test tubes were centrifuged at 5000rpm for 10 mints and serum was separated. Each serum sample was screened for the presence of anti-HCV antibodies by immunochromatographic technique (ICT) (Accurate®, USA). The positive samples were further processed for molecular detection and genotyping.

HCV-RNA extraction

HCV-RNA was extracted from serum using phenol-chloroform method as described previously with some modification (Petrelli *et al*., 1994). Briefly, 300µL of lysis buffer was added to 100µL of serum. Then 40µL of 3M sodium acetate (pH 5.2) along with 400µL water saturated phenol and 80µL of chloroform were added to the same tube. The tube was vortexed gently and centrifuged at 14,000 rpm for 15–20 minutes. The upper phase was transferred into another tube and 0.3mL isopropanol was added. The tube was vortexed shortly and kept at –20ºC for 15-20 minutes. After centrifugation, the supernatant was discarded, and RNA pellet was washed twice with 70% ethanol and vacuum dried.

cDNA synthesis

RNA pellet was used for complementary DNA (cDNA*)* synthesis using the reverse transcriptase enzyme as described earlier (Shafique *et al*., 2009). The cDNA was synthesized by gene specific reverse transcriptase antisense primer; Moloney murine leukemia virus (M-MLV) (AS1: 5'-GTGCACGGTCTACGAGACCT-3') and incubated at 42°C for 1 hour. The cDNA was stored at –20°C.

Amplification of cDNA by regular PCR

Regular PCR was performed using the synthe-sized cDNA as a template with the following prim-ers; (F-1:5'-GCCATGGCGTTAGTATGAGT-3') and

(R-1:5'-GTGCACGGTCTACGAGACCT-3') by following thermocycler conditions; denaturation at 94°C for 3 minutes, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds with 30 cycles.

Amplification of regular PCR product by nested PCR

Nested PCR was carried out using 1-2µL of regular PCR product with following internal primers; (F-2: 5'-GTGCAGCCTCCAGGACCC-3') and (R-2: 5'-CCGTGAGCGTTCGTGGGATA-3'). Amplicons were electrophoresed on 1.5% agarose gel and bands were visualized using Gel Doc system under UV light.

HCV genotyping

HCV genotypes were determined by Restricted Fragments Length Polymorphism RFLP using amplified products as described by Ahmad *et al*. (2007). Briefly, three sets of restriction enzymes; Set-I: (HaeIII and RsaI), Set-II: (HinfI and ScrFI) and Set-III: (HinfI and MvaI) were used. Moreover, the restricted PCR products were separated on 4% agarose gel and HCV genotypes were identified based on PCR-RFLP analysis.

HCV genotyping using type specific primers

HCV genotyping was also performed using type-specific primers as mentioned by Ohno *et al*. (1997). In short, core gene specific primers were used for the amplification of core region and multiplex PCR was performed for genotype identification using the following primers; (Sc2: 5'-GGGAGGTCTCGTAGACCGTGCACCATG-3'), (Ac2: 5'- GAG (AC) GG (GT) AT (AG) TACCCCATGAG (AG) TCGGC-3'). Mix-1 primers; (S7: 5'-AGACCGTGCACCATGAGCAC-3'), (S2a: 5'-AACACTAACCGTCGCCCACAA-3'), (G1b: 5'-CCTGCCCTCGGGTTGGCTA(AG)-3'), (G2a: 5'-CACGTGGCTGGGATCGCTCC-3'), (G2b: 5'-GGCCCCAATTAGGACGAGAC-3'), (G3b: 5'-CGCTCGGAAGTCTTACGTAC-3'), Mix-2 primers; (S7:5'-AGACCGTGCACCATGAGCAC-3'), (G1a:5'-GGATAGGCTGACGTCTACCT-3'), (G3a:5'-GCCCAGGACCGGCCTTCGCT-3'), (G4: 5'-CCCGGGAACTTAACGTCCAT-3'), (G5a: 5'-GAACCTCGGGGGGAGAGCAA-3'), (G6a: 5'-GGTCATTGGGGCCCCAATGT-3').

Thermal cycle conditions were: denaturation at 94°C for 5 minutes, annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds. PCR products were electrophoresed on 2% agarose gel.

RESULTS

Out of 3200 clinical samples, 172 (5.37%) samples were HCV positive by ICT method. Among these, 96 (55.8%) samples were confirmed by PCR (Fig. 1). On the basis of clinical history of each patient, they were divided into four groups such as acute, chronic, cirrhotic and hepatocellular carcinoma (HCC). Most of the HCV positive cases (25%) have been identified in 41-50 years of age group, followed by 22.9% in 31-40 years and 20.8% in 51-60 years. Moreover, most of the HCV patients belong to the Group I (n=12) followed by Group III (n=11) and Group IV (n=09) as showed in Table I.

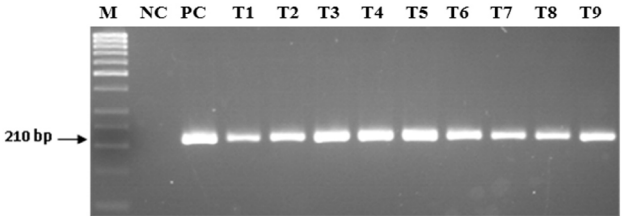

Fig. 1. the HCV-RNA positive samples. M-50bp ladder. NC,negative control; PC, positive control; T, tested samples.

Table I. Distribution of HCV patients in various age groups.

Age (years)	Group I	Group II	Group III	Group IV	(%)
10 - 20	00	03	00	00	3.1
21 - 30	08	08	01	00	17.7
31 - 40	12	08	02	00	22.9
41 - 50	04	06	11	03	25.0
51 - 60	03	02	06	09	20.8
61 - 70	00	00	03	07	10.4
Total	27	27	23	19	

Group I: Acute hepatitis C, Group II, Chronic hepatitis C; Group III, HCV cirrhosis; Group IV, HCV hepatocellular carcinoma.

The distribution of HCV is more common in male than female individuals (data not shown) as we reported in an earlier study describing the higher prevalence of HCV in males 77.33% (232/300) than in females 22.66 (68/300), however, there was no significant difference found in the distribution of HCV genotypes among males and female individuals (Ahmad *et al*., 2007).

HCV genotyping by RFLP

HCV genotypes were determined by RFLP analysis of PCR confirmed samples as described by Ahmad *et al*. (Ahmad *et al*., 2007) using three sets of restriction enzymes as shown in Figure 2 and Figure 3. The RFLP data demonstrated that HCV genotype 3 was found to

be the most prevalent genotype in these tested samples followed by genotype 1. However, multiplex PCR based genotyping method provides more detailed information about the distribution of HCV subtypes.

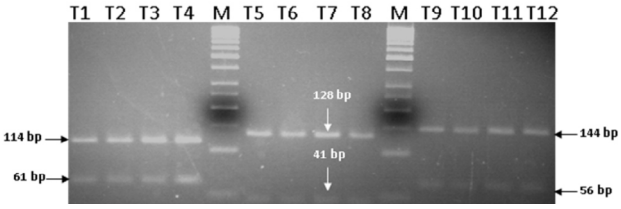

Fig. 2. HCV genotyping by RFLP. M-50bp ladder, T1-T4: tested samples digested with HaeIII and RsaI, T5-T8: tested samples digested with ScrfI and HinfI and T9-T12: tested samples digested with MvaI and HinfI.

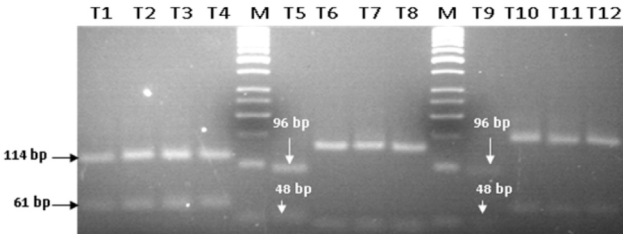

Fig. 3. HCV genotyping by RFLP. M-50bp ladder, T1, T5, T9: showing genotypes 1. T2, T3, T4, T6, T7, T8, T10, T11 and T12: tested samples showing genotype 3.

HCV genotyping using type specific primers

HCV RNA positive samples (n=96) were genotyped/subtyped using type specific primers. Among these tested samples, genotype 3A was the most predominant genotype i.e. 78.12 % (n=75) in all groups. More specifically, genotype 3A was 28.13% (n=27) in group G-I, 28.13% (n=27) in the group G-II, 11.5% (n=11) in the group G-III and 10.4% (n=10) in the group G-IV. However, 3.8% (n=3) samples showed mixed HCV genotypes i.e. 3a/2a, 3a/3b and 3a/1a (Table II). A total of 18 samples (17.9%) were found with untypable genotypes, as no genotype-specific PCR fragments bands were observed in these samples (Fig. 4 and Fig. 5).

DISCUSSION

HCV is a major health issue throughout the world, mainly in developing countries like Pakistan. There is the highest burden of HCV present in Pakistani population, such as in the present study, preliminary 172 (5.37%) suspected clinical samples were positive for HCV while 96 (55.8%) were further confirmed by PCR. Few studies reported the HCV prevalence 6.2% and 8.64% (Arshad and Ashfaq, 2017) in general Pakistani population. Moreover, a limited data is also available about the prevalence of HCV in various cities of Pakistan such as in Faisalabad (28%), Gujranwala (23%) and Lahore (16%) (Ahmad *et al*., 2007).

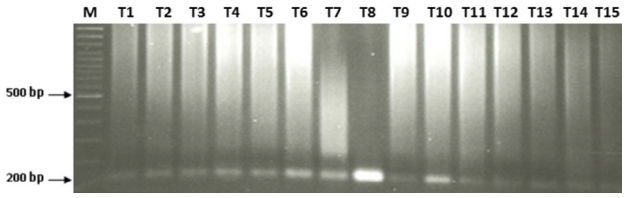

Fig. 4. Multiplex PCR with Mix-A of RT-PCR positive samples. M-50bp ladder, T1-T15: tested samples.

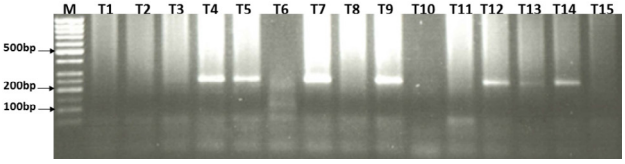

Fig. 5. Multiplex PCR with Mix-B of RT-PCR positive samples. M-50bp ladder; T4, T5, T7, T9, 12, 13 and 14: Amplified products with band size 232bp.

Table II. Prevalence of HCV genotypes in four different groups (n=96).

Subject groups	Total genotypes (n)	Genotype 3a (n)	Mixed genotypes (n)
Group-I (n=27)	27	27	00
Group-II (n=27)	27	27	00
Group-III (n=23)	13	11	02 (3a+2a)(3a+3b)
Group-IV (n=19)	11	10	01 (3a + 1a)
Total	78(81.25%)	75(78.12%)	3 (3.13%)

For group details, see Table I.

In the current study, results were analyzed to determine the prevalence of HCV infection in different age groups. The high prevalence of HCV (25%) has been found in 41-50 years age group followed by 22.9% in 31-40 years (Table I). Generally, this data agrees with previous reports describing that the most affected age range of patients found was 21-40 years as compared to the teenage and older age groups (Ahmad *et al*., 2010). However, in United States, HCV prevalence is the highest in 30-49 years old individuals who account for two-thirds of all infections, and lower than average among persons < 20 and > 50 years old (Alter, 2007; Armstrong *et al*., 2006). This pattern indicates that the most HCV transmission occurred in last

20-40 years, and primarily among young adults, a pattern similar to that observed by others (Law *et al*., 2003).

HCV has great heterogeneity that has increased with the passage of time due to low fidelity and error prone nature of HCV RNA dependent RNA polymerase enzyme. HCV has evolved into different genotypes, currently; it has been classified into seven major genotypes and 67 subtypes (Smith *et al*., 2014). In the present study, PCR positive samples were further tested for genotyping and genotype 3A was found to be the most predominant genotype i.e. 78.12 % (n=75). However, 3.8% (n=3) samples showed mixed HCV genotypes. The data showed that the most prevalent HCV genotype was 3 followed by genotype 1 that agrees with few earlier reports in HCV patients from Faisalabad and Rawalpindi / Islamabad cities, Punjab province of Pakistan (Ahmad *et al*., 2007; Afzal, 2017). Moreover, the presence of a high percentage of HCV genotype/ subtype 3a in this study, i.e. 78.12 % (n=75) is also in accordance with some previous studies (Waqar *et al*., 2014; Aziz *et al*., 2013; Butt *et al*., 2010; Idrees *et al*., 2009; Waheed *et al*., 2009; Ahmad *et al*., 2010; Ahmad *et al*., 2007). However, 3.13% tested samples showed mixed HCV subtypes i.e. 3a/2a, 3a/3b and 3a/1a that also supports to few early studies (Attaullah *et al*., 2017; Butt *et al*., 2011). The presence of mixed genotype infections in HCV infected patients could be due to multiple risk factors associated with HCV transmission i.e. intravenous drug users, blood transfusions and the use of blood products, multiple use of needles/syringes, sharing razors during shaving or circumcision, piercing instruments, nail clippers, and toothbrushes and major or minor dental surgery (Butt *et al*., 2011). Moreover, we also found 17.9% (n=18) samples with untypable genotypes, as no genotype-specific PCR fragments band was observed in these samples that was also in concordance with earlier reports (Waqar *et al*., 2014). However, we couldn't determine HCV genotypes from all RT-PCR positive patients' sera. This might be the reason that these sera may possess genotype(s) that is novel and we couldn't able to detect it with the present assay or may have low viral load also agrees to a previous study by Groopman *et al*. (Groopman *et al*., 2008).

This study has reported that among mixed genotypes, subtype 3a alone or in combination with other types is more prevalent in accordance with previously published data that genotype 3a is the major subtype as it needs a shorter duration of treatment as compared to other genotypes. However, there is a limitation exists in our interpretation of the results that we do not have the facilities to sequence the untypable genotypes in the study. Thus, there is a possibility of existence of some novel genotypes/subtypes that could not be detected by the present available techniques which may lead to severity of the disease.

CONCLUSIONS

This study concluded a high spread of HCV in our clinical settings and genotype 3a is more prevalent. The genotype distribution may have geographic in origin and severity of disease may be associated with some other risk factors. HCV infection is becoming a public health issue in Pakistan and huge efforts need to be taken by public health authorities to educate the general population about the prevention and the importance of early detection and start of therapy. Thus, there is an urgent need to have a cost-effective and reliable in a house HCV genotyping system that could be used for clinical and epidemiological studies for the local population in Pakistan.

Statement of conflict of interest

The authors declare that there is no conflict of interest.

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