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Diagnosis of Hepatitis C virus and its different Genotypes; A Qualitative Approach

Muhammad Aetesam Nasir[1], Faisal Muzaffar[2], Muhammad Waqar Mazhar[3]*, Abdus Salam[3], Muhammad Imran[3], Hadia Fatima[3], Mariyam Saleem[3], Amber Maria[3], Muhammad Naeem[3] and Muhammad Sharjeel[3]

[1]HITEC Institute of Medical Sciences, Taxila, Rawalpindi, Punjab

[2]Multan Medical and Dental College, Jahangirabad Shah Rukn E Alam Town, Multan, Punjab 60000

[3]Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Punjab, Pakistan,

***Corresponding Author:** Muhammad Waqar Mazhar, Department of Bioinformatics and Biotechnology Government College University Faisalabad, 38040, Punjab, Pakistan*

E-mail: waqarmazhar63@gmail.com

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ABSTRACT

Hepatitis C belongs to the family of Flaviviridae and it causes the most serious viral hepatitis infections. Clinical problems ncirrhosis, chronic hepatitis, and hepatocellular carcinoma due Hepatitis C virus (HCV). The HCV genotype frequency in Pakistan is still missing, therefore it's very important to investigate the HCV genotypes in infected patients. Molecular techniques play a very important role in the diagnosis of viral infection. In this study, we detect the different genotypes of HCV by using Polymerase Chain Reaction with the help of specific primers., firstly the samples were collected from different areas of Faisalabad and DNA extraction was performed. Then PCR was performed by using specific primers. After that, the PCR-positive samples were used for genotyping by using a methodology with minor changes.

This study shows the different genotypes of 2a, 1b, and 3a. The most prevalent genotype was 3a, 1b, and 2a with 60%, 20%, and 10% respectively. remaining samples were untypeable and investigate for further analysis. For further analysis and epidemiology, sequencing of these genotypes was useful in diagnosis.

Keywords: HCV, Virus, Pakistan, Flaviviridae Family.

INTRODUCTION

Hepatitis C virus (HCV) is the second most common factor that causes acute hepatitis and it belongs to the Flaviviridae family and Hepacivirus genus [1,2]. In Pakistan, is a global health problem for people and Pakistan has a higher frequency rate other than countries like Nepal 1.0%, India 0.66%, China 1%, Iran 0.87%, Myanmar 2.5%, and Afghanistan 1.1% [3]. HCV has hepatotropic and lymphotropic properties and a complete genome. HCV has a 9.4 kb genome size and positive sense RNA. The sequence contained a 5′ UTR region that contains 341 base pairs, an open reading frame that contains 3011 amino acid coding for the poly-peptide, and a 3′ UTR region that contains 27 base pairs [4]. The antisense RNA OF HCV resemble arthropod-borne viruses, with three N-terminal protein C, E1, NS2, and The four terminal proteins NS2.NS4, NS5, and NS3 play an important in viral replication in the human body. The most highly conserved region present in 5′ UTR of HCV RNA [5] (Figure1).

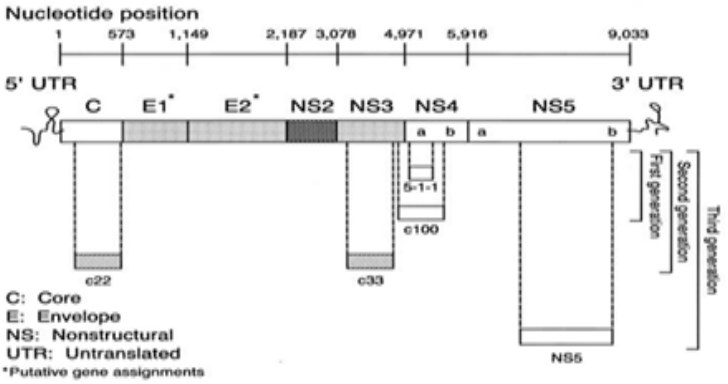

Figure 1. Genomic organization of HCV

In 80% of chronically infected individuals about one-fifth goes on to develop cirrhosis in 15 years to -20 years with still diffused symptoms [6]. If the cirrhosis becomes severe, the patient reaches an end-stage liver disease where it needs liver transplantation. In 1%-4% of cirrhosis cases, the patients develop liver cancer and death as a possible outcome (Figure 2) [7].

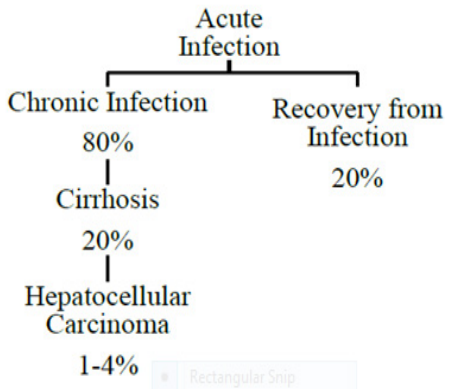

Figure 2. The possible outcome of the HCV infection

Worldwide, due to HCV 130 million, -200 million people are infected and every year more than 350,000 people are died due to hepatic viral infection [8]. A higher prevalence of hepatitis was reported in Eastern Asia and Africa. In Egypt, the prevalence of hepatitis is higher compared to other countries, 14% of people are infected with Hepatitis C [9] (Figure 3).

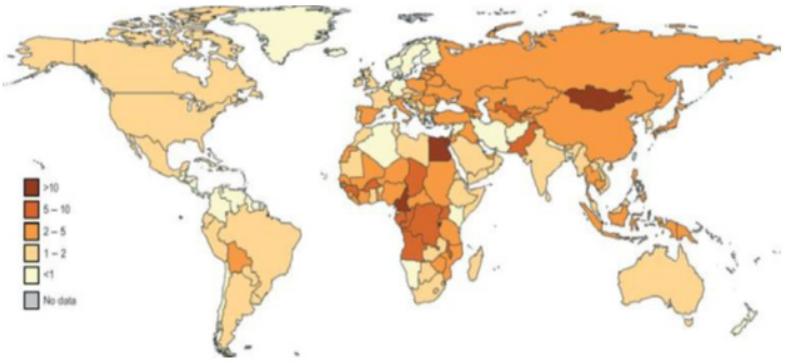

Figure 3. Geographical Distribution of hepatitis C virus

METHODOLOGY

Sampling

Blood samples were collected in labeled plasma collection tubes, from both male and female patients of Faisalabad and its surrounding villages. The serum was separated after complete centrifugation (4000 rpm for 10 minutes) within an hour of venipuncture.

RNA extraction

RNA was extracted from 160 µL of serum by usingFavorPrep™ Viral Nucleic Acid Extraction kit.

cDNA synthesis

cDNA was synthesized by using the Revertaid H-Minus First strand cDNA synthesis kit (Fermentas). According to the protocol of the manufacturer. Performed nested PCR and then band visualized under gel electrophoresis (Table 1).

Table 1. PCR Profile

Steps	Temperature °C	Time	Number of cycles
Initial denaturation	94.5 °C	4 min	1
Denaturation	95 °C	32 sec	30
Annealing	61 °C	32 sec	
Extension	72 °C	42 sec	
Final extension	72 °C	6 min	1

HCV-specific genotype PCR

Genotyping was performed by using specific primers that detect 9 subtypes of HCV ((1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a). Genotype-specific primer was performed like the first round of PCR (Tables 2 and 3).

Table 2. Primers used for genotyping (Mix A).

Primer	Sequence (5′–3′)	Nucleotide position
S7	AGACCGTGCACCATGAGCAC	-12–8
S2a	AACACTAACCGTCGCCCACAA	40–60
G1b	CCTGCCCTCGGGTTGGCTA(AG)	222–203
G2a	CACGTGGCTGGGATCGCTCC	178–159
G2b	GGCCCCAATTAGGACGAGAC	325–306
G3b	CGCTCGGAAGTCTTACGTAC	164–145

Table 3. Primers used for genotyping (Mix B).

Primer	Sequence (5′–3′)	Nucleotide position
S7	AGACCGTGCACCATGAGCAC	-12–8
G1a	GGATAGGCTGACGTCTACCT	196–177
G3a	GCCCAGGACCGGCCTTCGCT	220–211
G4	CCCGGGAACTTAACGTCCAT	87–58
G5a	GAACCTCGGGGGGAGAGCAA	308–289
G6a	GGTCATTGGGGCCCCAATGT	334–315
The S stands for sense stands and A/G stands for antisense in the naming of primers		

RESULTS

RNA Extraction

Eighteen Blood samples were collected from both male and female infected individuals from different areas of the Faisalabad region. RNA was extracted from these samples and cDNA was synthesized which were labeled as 1 to 18. After that, samples were stored at – 20 °C till the polymerase chain reaction.

Identification of Hepatitis C virus by Polymerase Chain Reaction (PCR)

PCR reaction was performed with HCV-specific primers. PCR results are shown in Figure 4. In Figure 4, lane no. 1 to 18 represents PCR results of different samples, lane M represents the positive control, lane N is the negative control and M is the 1 kb DNA ladder. PCR results were positive for 10 samples with a fragment size of approximately 250 bps. After that, the DNA of HCV-positive samples was used to diagnose the HCV genotype of these samples.

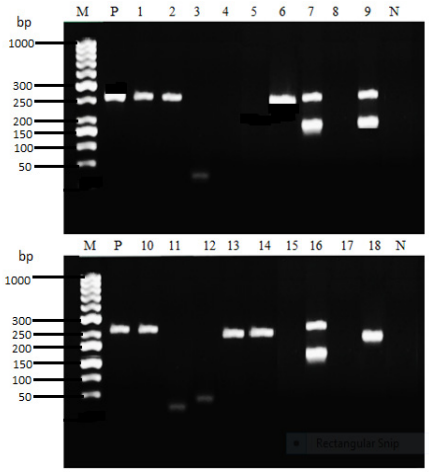

Figure 4. Agarose gel electrophoresis of PCR with HCV-specific primers. M: 1kb DNA ladder. 1-18 lanes are the PCR results of all samples. PCR results are positive in lanes no.1,2,6,7,9,10,13,14,16,18. P: Positive controls. N: Negative controls

Identification of HCV genotype

The 3a genotype (60.0%) of HCV was detected in the positive population and its more prevalent genotype in selected areas. Genotype 1b was observed in 2 samples (20%) followed by genotype 2a which was observed only in 1 sample (10%). Other HCV genotypes were not detected in the positive sample. The genotype of one sample (10%) was not determined and was declared as untypeable by the method described (Figures 5 and 6).

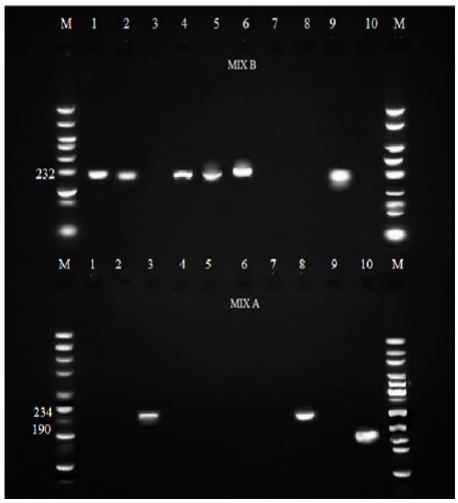

Figure 5. Different HCV Genotypes PCR products pattern analyzed under gel electrophoresis. M: 1kb DNA ladder. 1-10 lanes (Both in mix A & B) are the HCV genotype results of all samples. In lanes 1, 2,4,5,6 and 9, no specific product is detected with mix B (lower panel), but a specific band of 232 bp is seen with mix B. Similarly, HCV genotype 1b samples showed only two bands of 234 bp with mix A (lanes 3 and 8), while genotype 2a showed a specific band of 190 bpin mix A (lane10).

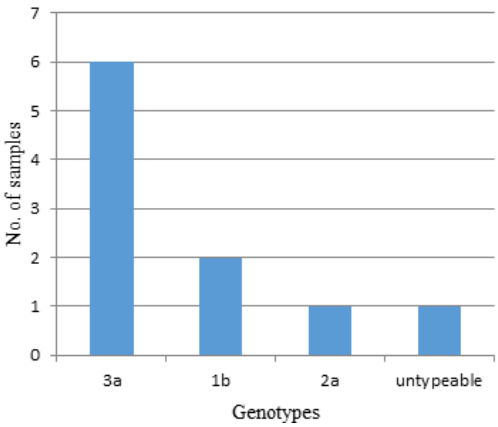

Figure 6. Hepatitis C virus (HCV) genotypes

The final results of HCV genotypes are in the following table see Table 4.

Table 4. Summary of final results

Sr. No.	Sample No.	PCR Results	Amplified fragment size	Genotype
1	1 G	Positive	232	3a
2	2 G	Positive	232	3a
3	3 G	Positive	234	1b
4	4 G	Positive	232	3a
5	5 G	Positive	232	3a
6	6 G	Positive	232	3a
7	7 G	Positive	_	Untypeable
8	8 G	Positive	234	1b
9	9 G	Positive	232	3a
10	10 G	Positive	190	2a

DISCUSSION

HCV has come to the top of virus-induced liver diseases in many parts of the world and has gained endemic proportions in our population but there is no national data collection system for evaluation of genotypes of HCV infection. Identification of the infecting virus genotype is important because it provides information as to strain variation and potential association with disease severity [10,11]. A suitable and reliable HCV genotyping method is inevitable for large-scale epidemiological and experimental studies. In addition, it is of epidemiologic value because it sheds light on whether prevalent HCV strains are similar to that endemic in a certain region, such as here in South Asia.

Several laboratory procedures aimed at identifying HCV genotypes have been described. HCV genotype determination in full-length genomic sequence analysis followed by phylogenetic analysis is still the golden standard [12]. Though this system is expensive and time-consuming and cannot be adapted to clinical studies or extensive standard use [13]. PCR has been broadly used for genotyping which is based upon the amplification of virus sequences in clinical specimens, using type-specific primers that specifically amplify different genotypes [1,14]. Information obtained from various parts of the world has focused on the increasing implication of HCV genotyping and stressed the need for easy, reliable, cost-effective, and fast techniques for mass screening.

Hepatitis C is also common in Pakistan but accurate epidemiological information is quite limited. In the outer edges of the cities and remote areas, unqualified medical and dental practitioners, lady health visitors, midwives, and barbers often use unsterilized instruments which are major potential sources of spreading HCV infection in the urban and rural population of Pakistan [15].

HCV has six major genotypes and hundreds of subtypes globally identified so far [16]. Besides distinct geographic distributions and alterations in pathogenesis exhibited by different genotypes, the response to therapy also varied between them [17].

Our data showed that genotype 3a (60%) followed by genotype 1b (20%) is prevalent among the studied population. Idrees and Riazuddin, also reported that HCV genotype 3a was found predominant in the general population of Pakistan [18]. This showed reduced diversity of subtypes in Pakistan. It would therefore appear from different studies conducted in different parts that substantial regional differences do exist in our country. Genotype 1 is the second-highest genotype in the country. Baluchistan shares a long border with Iran in the west where genotypes 1a and 3a are most prevalent [19].

A high prevalence of variants of genotype 3 alone is over 79.43% in Pakistan alone or combination with another genotype. The high prevalence of HCV genotype 3 in Pakistan is good hope for a cure as well as control of HCV infection [20]. Genotype 3 requires a shorter duration of treatment as compared with genotype 1, with its associated reduced cost and side effects. The predominance of HCV genotype 3 in our population confirmed the predominance of HCV genotype 3 in the surrounding countries including India, and Iran [21].

Previously, a proportion of patients will have their genotype reported as untypeable (3.306%). The presence of untypeable samples indicated that some novel genotypes are present in Pakistan [22]. For being able to identify untypeable genotypes, phylogenetic analysis of extensive genome sequences is required and continuous efforts are required toward a better characterization of this variant.

HCV genotyping provides valuable epidemiological and therapeutic information. Current therapy, which consists of a combination of pegylated interferon and ribavirin, gives a response rate of between 48% (genotypes 1, 4, 5, and 6) and 88% for genotypes 2 and 3 [23]. The duration of therapy is variable as well for different genotypes. These findings indicate the importance of genotype knowledge before therapy.

Pakistan is among the worst affected nations and carries one of the highest burdens of HCV. The literacy rate is very low and there is a lack of an effective disease awareness system due to which the general public is least educated about the pathogenicity, routes of transmission and the proper procedures of diagnosis and treatment. A considerable portion of the population lives on less than a dol-

lar a day. HCV infection, therefore, has become an economic burden on the impoverished people of Pakistan and especially in Punjab and KPK.

HCV is known as the silent killer because in the majority of the cases no proper signs or symptoms are visible in the early stages of infection and when symptoms appear then the treatment is difficult. Secondly, in most developing countries diagnosis is not proper due to a lack of facilities. In the absence of an effective vaccine, efforts should be focused on preventive strategies to reduce HCV transmission, including universal screening of blood and blood products, proper sterilization of medical and dental equipment, mandatory use of disposable needles, avoidance of unnecessary injections or procedures, and needle-exchange programs for injecting drug users. Additionally, health workers (especially in developing countries) and the public should be educated about the risk of infection from unsafe practices, and individuals at risk should be counseled and tested for HCV. Treatment of HCV, however, is unlikely to have a major effect on the epidemiology of HCV since most infected individuals in the world remain undiagnosed and have no access to expensive medications.

The present study shows that the distribution of HCV genotype 3 in Faisalabad is similar to that in other areas of Pakistan. HCV types 3 and 1 are prevalent in this area which can better respond to interferon therapy but types 2 and 4 were also circulating and needed longer treatment. Proper epidemiological studies and treatment strategies should be initiated in this area. Appropriate preventive measures should be also taken into consideration to control the spread of this dreadful disease.

CONCLUSION

Multiple genotypes of HCV have been isolated throughout the world. The identification and characterization of HCV types and subtypes have major implications for HCV vaccine development. It is clear that HCV genotypes are important epidemiologic markers and may alter the sensitivity and specificity of diagnostic assays for the detection of HCV. HCV genotyping in combination with other markers, such as quantitative evaluation of HCV RNA, may be beneficial in the management of chronic hepatitis C and the selection of candidates for interferon treatment. Genotype determination could potentially be used to decide the length of treatment. More studies are needed before guidelines can be established for the routine use of genotyping outside clinical trials and research laboratories

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