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Phylogenetic Characterization of the 5′ Untranslated Region of Untypable HCV Genotypes Circulating in Pakistan

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HCV · Genotyping · 5′ UTR · Sequencing · Phylogenetic analysis

Abstract
Introduction: Commercial methods for HCV genotyping is challenged by the increased prevalence of untypable genotypes in Pakistan. ***Objective:*** The aim of the current study was to perform nucleotide sequencing of 5′ UTR region for genotyping of viral isolates circulating in Peshawar, Pakistan. ***Methods:*** The total number of commercially untypable samples were 94 in which 18 samples were sequenced for the characterization of 5′ UTR region. Post-sequencing analysis was performed for genotype identification ($n = 18$) and molecular phylogenetic analysis. ***Results:*** The current study reveals different genotypes, that is, 10/18 viral isolates were found to be genotype 3a (55.55%), 3 isolates (genotype 3b, 16.66%), 2 isolates (genotype 6h/6g, 11.11%), 2 (6g/d, 11.11%), and 1 sample (genotype 1c, 5.55%). In addition, genotype 3a is the dominant representative of HCV circulating in Pakistan and has been increasing across the country. ***Conclusion:*** The current study also reveals that genotype 6 (2 were genotype 6h/6g and 2 were 6g/d) is also circulating in Pakistan and not restricted to South China and Hong Kong.

Introduction

Viral hepatitis infection is a major causative agent of chronic and acute liver diseases worldwide. In Pakistan, hepatitis C is one of the major health threats and approximately infecting 6% of the population [1]. The chronic infection has high risk of developing other liver complications, that is, hepatosteatosis, hepatocellular carcinoma, liver fibrosis, and liver cirrhosis [1]. HCV is an enveloped virus with a positive-sense RNA molecule of approximately 9.6 kb and is classified into the *Hepacivirus* genus within the *Flaviviridae* family of viruses [2, 3].

HCV isolates exhibit significant genetic heterogeneity globally. Previously, 7 genotypes (genotype 1–7) of HCV have been identified, which differ by 31–33% at the nu-

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Table 1. Primers used for HCV qualitative detection targeting 5′ UTR region

S/No.	Primer code	Primer sequence (5′–3′)	Primer length	Nucleotide position
1	Outer sense	CTGTGAGGAACTACTGTCTT	20	45–64
2	Outer antisense	ATACTCGAGGTGCACGGTCTACGAGACCT	29	349–320
3	Inner sense	TTCACGCAGAAAGCGTCTAG	20	63–82
4	Inner antisense	CACTCTCGAGCACCCTATCAGGCAGT	26	313–288

cleotide and deduced amino acid level over the complete genome. Moreover, there are several subtypes (a, b, c, etc.) which differ by 20–25% on the nucleotide level and inferred amino acid level from one another [4]. Recently, HCV genotype 8 has been reported in India in 4 patients [5]. Genotypes 1, 2, and 3 appear to have a worldwide distribution, and 1a and 1b are the most common genotype in the Europe [6] and the USA [7]. Genotype 3 is more prevalent in South Asia (Afghanistan, Pakistan, Bangladesh, India, and Nepal). In Pakistan, 3a is the leading genotype (60.3%) followed by 2a and untypable genotypes [8].

Strain or genotype-specific antiviral treatment of patients requires accurate HCV genotyping. Efficacy of antiviral drugs does not remain the same against all genotypes of HCV; it varies from 1 genotype to another. It has been observed that sustained virological response to interferon plus ribavirin combination therapy was 87.5% in patients infected with HCV-3 genotype. On the other hand, sustained virological response to the same therapy was reported as 24.3% in patients with HCV-1 genotype [9]. Advanced methods for viral genotyping are by using the direct sequencing of specific amplified genome fragments of the virus (i.e., 5′ UTR, core, E1, and NS5B regions) [10–12]. Commercial genotyping assays that target the 5′ UTR are widely used by clinical laboratories for assessing the different HCV genotypes [13]. However, in Pakistan mostly conventional methods are being used for this purpose. These methods detect genetic variation (in terms of size of amplified product) on agarose gel, and a great ratio (up to 16.5 to >30%) of samples remains untypable during routine diagnostics. Presence of such large number of untypable samples needs nucleotide sequence-based studies. The current study was performed to identify the genotypes and subtypes of untypable samples on the sequence-based phylogenetic analysis of 5′ UTR region that will be helpful in diagnosis, epidemiology, and prescribing therapy for patients.

Materials and Methods

Patient's Samples

HCV-infected serum samples were collected from Abaseen Research and Reference Laboratory, and different public sector hospitals (Khyber Teaching Hospital, Hayatabad Medical Complex, and Lady Reading Hospital), Peshawar, Khyber Pakhtunkhwa (KP), Pakistan. The samples were collected with the detailed (signed) consent from the patients. The designed proforma/questionnaire contains all information (such as name, age, gender, transmission route, and place of living). Males and females both were included in this study. A total of 332 samples were included for the proposed research.

Study Design

This study was designed to amplify 5′ UTR region of untypable HCV isolates and then sequenced the amplified product. For this purpose, PCR amplification of 5′ UTR region was performed after performing Ohno's genotyping method. Evolutionary analysis of the resultant 5′ UTR product after sequencing was performed.

Inclusion Criteria

Only samples that were positive for HCV-RNA by PCR and that were untypable upon Ohno's genotyping were included in the present study.

Exclusion Criteria

Those samples that were successfully genotyped with Ohno's method were excluded from the study. Lack of required information, small sera samples, patients having coinfection with other hepatitis viruses, and patients previously treated (antiviral therapy) for HCV were excluded from the study.

Primers for Qualitative PCR Amplification

The qualitative PCR for HCV was done utilizing the method and primers already described by Ohno et al. [14] with little modification. The details of the primers used in the current study are listed in Table 1.

Viral RNA Extraction, Reversed Transcription, and HCV 5′ UTR Amplification

HCV-RNA was extracted from 150 μL serum using (FAVORGEN Viral nucleic acid extraction kit: Catalog No. FAVNK 001-2) according to the manufacturer protocol and resultant HCV-RNA was stored at −70°C. 10 μL RNA was used for cDNA synthesis with outer antisense primer (10 pmol/μL) using Moloney murine leu-

Table 2. Gender-wise distribution (n = 322)

Genotypes	Subtypes	Male	Female	Total	Percentage
1	1a	7	0	7	2.2
	1b	9	12	21	6.5
2	2a	3	0	3	0.9
	2b	7	9	17	5.0
3	3a	57	72	129	40.1
	3b	15	12	27	8.4
4	4	6	16	22	6.8
Untypable		46	48	94	29.2
Mix		3	0	3	0.9
Total		153	169	322	100

Table 3. Age-wise diversity of HCV

Age, years	Genotypes							Untypable	Mix	Total	Percentage
	1a	1b	2a	2b	3a	3b	4				
15–30	3	10	0	6	41	12	7	34	2	115	35.7
31–45	3	4	1	7	54	6	3	43	1	122	37.8
46–60	1	6	2	3	30	9	12	17	0	80	24.9
<60	0	1	0	0	4	0	0	0	0	5	1.6
Total	7	21	3	16	129	27	22	94	3	322	100

kemia virus reverse transcriptase enzyme (M-MLV RTase, Invitrogen). Nested PCR amplification protocols were employed for qualitative detection of HCV. For detection, the products were electrophoresed on 2% TAE agarose gel.

Genotyping

HCV genotypes 1–6 were identified using specific primers for core region of HCV as described previously by Ohno et al. [14].

Interpretation of Untypable HCV 5′ UTR Partial Nucleotide Sequence

For sequence analysis, amplified product of the 5′ UTR region of 20 selected isolates was purified with gel extraction kit (VIVANTIS) and was used as templates for sequencing in the Big-Dye Terminator cycle sequencing ready extraction kit (Applied Biosystems). Sequencing was done on ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., 850 Lincoln Center Drive, Foster City, CA, USA). Out of the total 20 untypable HCV genotype, 18 samples were successfully sequenced. To determine the HCV genotype of untypable, all the sequences were then assessed using NCBI-BLASTN's software (online version).

Phylogenetic Analysis

Phylogenetic analysis of untypable HCV isolates was performed with MEGA-6 software [15]. Neighbor-joining method was used to assess evolutionary history of the isolates [16]. The analysis involved 18 sequences from the current study and 115 reference sequences from GenBank.

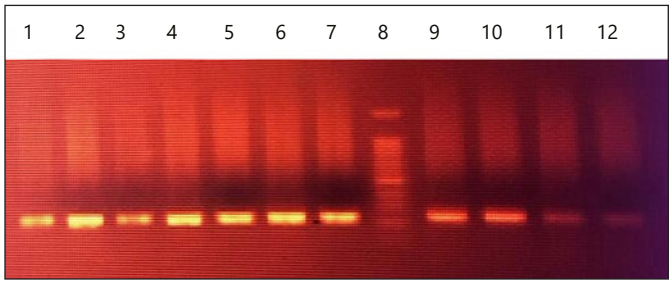
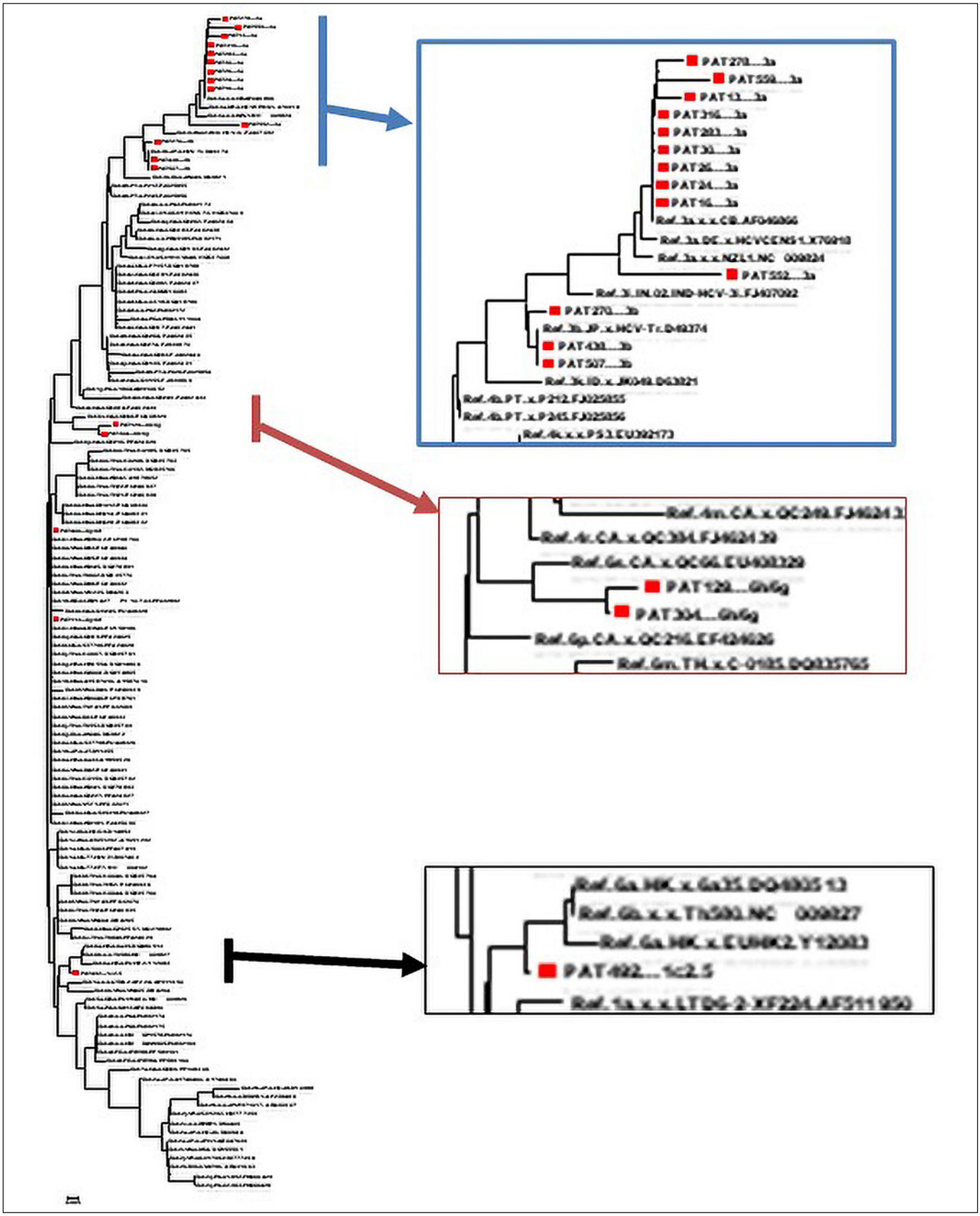


Fig. 1. Agarose gel photograph showing HCV 5′ UTR amplified products. Lane 8 represents 100-bp DNA marker, Lanes 9 and 10 showing positive control 251-bp specific fragments, and Lanes 1–7 present positive samples HCV-specific bands of 251-bp.

Results

A total of 322 patients were included, of which 52.5% (n = 169) were females and 47.5% (n = 153) males and were of different ages from 18 to 65. Seven individuals have subtype 1a, 21 have subtype 1b, 3 have subtype 2a, 17 have subtype 2b, 129 have subtype 3a, 27 have subtype 3b, and 22 have genotype 4. Rest of the 94 samples were untypable and just 3 individuals had mixed genotypes in-



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fection. Out of 94 untypable samples, 20 samples were selected for sequencing in which 18 samples were successfully sequenced for the 5′ UTR region. The agarose gel picture representing 5′ UTR region bands of HCV is illustrated in Figure 1. The demographic characteristics of all the enrolled patients in the present study are given in Tables 2 and 3.

Evolutionary Analysis

The evolutionary analysis (Fig. 2) of the 18 sequences shows that HCV new subtypes are equally emerging in the country, like Pakistan. This analysis shows that among all the isolates, genotype 3a is abundant followed by 3b genotype. Out of 18 samples, 10 were genotype 3a, 3 were genotype 3b, 2 were genotype 6h/6g, 2 were 6g/d, and only one patient was 1c.

Discussion

Frequency of HCV infection in Pakistani population is significantly higher as compared to the neighboring countries like Iran, India, Afghanistan, Myanmar, and Nepal [17]. In Pakistan since the last 2 decades, combination treatment with interferon and ribavirin continues to be used widely in routine practice in HCV-infected patients [18]. HCV genotyping is very important for diagnosis and treatment of HCV-infected patients as standard HCV antiviral therapy depends on viral genotypes [19]. The aim of the current research work was sequencing of 5′ UTR region of HCV-RNA from positive untypable samples belonging to different regions of Peshawar, Pakistan. This study will be helpful for molecular epidemiology, serological diagnosis, vaccine/targeted therapies, disease progression, and treatment response.

In the present study, all the 322 samples were genotyped, out of which 94 samples were tested untypable HCV variants. 5′ UTR region of 20 untypable samples (randomly selected among 94 untypable samples) was amplified and sequenced. The 18 samples were classified based on phylogenetic analysis of the 5′ UTR regions. Sequencing-based genotyping showed that 72% of the total 18 samples of the current study were genotype 3 (55.55% 3a and 16.66% 3b), 22.22% were genotype 6 (11.11% were 6h/6g, and 11.11% were 6g/d), and 5.55% were 1c. Our results show that 3a is the most dominant genotype in KP. The current study indicates predominance of genotype 3a in Pakistan (55.55% 3a) and this phenomenon has also been observed previously in multiple studies[20–22]. Genotype 6 and 1c subtype were not previously reported from KP province Pakistan. Presence of 1c subtype in this study is may be due to immigration of peoples from genotype 1 endemic countries. In our study, 4 samples were reported to be genotype 6 (2 were 6h/6g and 2 were 6g/d). Genotype 6 has been found restricted to South China [23], southern Taiwan, and Hong Kong [24]. Peoples of Pakistan and China are very close, and they visit frequently for different purposes mostly for trade, education, etc. The visits of peoples to each country may be possible reason for emergence of genotype 6 in Pakistani population. This leads to variation in HCV epidemiological pattern especially in areas with high rate of immigration/traveling.

HCV genotype is a prerequisite for pan-genotypic treatment, and HCV possesses a high genetic diversity due to nucleotide substitutions which were considered the main reason for inter-genotype variations [25, 26]. This shuffling genotype trend can change the epidemiological pattern of the viral infection and could result in appearance of untypable viral isolates (by existing genotyping techniques) in Pakistan.

To eradicate HCV infection in Pakistan, the issue of untypable genotypes needs to be addressed by sequencing-based genotype approach and molecular cloning techniques to meet the limitations of old diagnostic assays and possibly prevent HCV infection.

Fig. 2. Phylogenetic tree based on current study sequences (indicated by red boxes) as well as reference sequences representing all HCV genotypes retrieved from the NCBI database. The reference sequences are shown with their accession numbers.

Conclusions

Pakistan is a country where more than one genotypes of HCV are present, and previous studies showed that genotype 3a is the most common genotype in Pakistani population. Direct sequence analysis of 5′ UTR region of untypable HCV isolates reveals that this technique is an effective approach of viral genotyping as compare to other genotyping assays, that is, hybridization or restriction analysis because this technique provides more detailed sequence information. Although direct sequencing method is not an efficient method for identifying mixed genotypes, phylogenetic analysis of untypable HCV isolates shows that new HCV subtypes are emerging in Pakistan. Our results reveal that 5 new subtypes (i.e., 2 were genotype 6h/6g, 2 were 6g/d, and 1 sample was 1c) emerged in country that may be spread from other regions. We suggest large-scale study to find new ways dealing with un-

typable HCV isolates as it is serious health issue because without genotype identification, the treatment of HCV is very challenging.

Statement of Ethics

This study does not involve any human or animal object. Only serum samples were obtained from patients infected with HCV. Informed written consent was obtained from the patients (or their guardians in case of infants).

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

I.R. and M.A. conceived the study. U.G., S.B., and I.R. performed the experimental work. A.K., S.U., L.A., and U.G. drafted the manuscript. M.A., I.R., and L.A. revised the manuscript. All the authors read and approved the manuscript.

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